

PHYSICOCHEMICAL SURFACE PROPERTIES OF GRAM-POSITIVE COCCI

With special reference to group A streptococci

Håkan Miörner



Lund 1983

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Title and subtitle PHYSICOCHEMICAL SURFACE PROPERTIES OF GRAM-POSITIVE COCCI With special reference to group A streptococci			
Abstract The effect of specific binding of human serum proteins to bacteria on their surface properties was investigated using partition in polymer two-phase systems. Pretreatment of bacteria with human serum proteins showed that binding of proteins to specific surface receptor structures on gram-positive cocci changes the physico-chemical surface properties of the bacteria. Isoelectric points and surface hydrophobicity of gram-positive cocci were determined in polymer two-phase systems. The results obtained indicated that group A, C, and G streptococci have isoelectric points around pH 3.75. Staphylococci showed an isoelectric point of around pH 2. Several different experimental approaches all pointed at lipoteichoic acid (LTA) as the major cell wall component responsible for surface hydrophobicity of group A streptococci. The content of surface LTA was determined on whole group A streptococci using a novel method. A correlation was found between the surface hydrophobicity of group A streptococci and the content of surface LTA. The possible contribution of M protein to the surface hydrophobicity of group A streptococci was also investigated. The development of a new assay of type-specific M antigens on whole group A streptococci enabled a study of the antigenic part of the M-molecule <u>in situ</u>. The results obtained clearly indicated that either the type-specific or the antiphagocytic moiety of M protein contributes to the surface hydrophobicity of group A streptococci. Studies on the interaction between aggregated β_2-microglobulin (β_{2m}) and group A streptococci confirmed that β_{2m} binds to streptococcal M protein. The localization of β_{2m} on the surface of all nucleated human cells suggest that it could represent a specific binding molecule for the adherence of group A streptococci <u>in vivo</u>.			
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- V Miörner, H., and K. Nivenius Larsson.: Assay of type-specific M antigens on whole group A streptococci. *Eur. J. Clin. Microbiol.* In press.
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Abbreviations

β_2m	- β_2 -microglobulin
BSA	- bovine serum albumin
CP	- change of partition
cpm	- counts per minute
Ig	- immunoglobulin
LTA	- lipoteichoic acid
PBS	- phosphate buffered saline
PEG	- polyethylene glycol
P-PEG	- polyethylene glycol palmitate
RBC	- red blood cell
RIA	- radioimmunoassay
S-PEG	- polyethylene glycol sulfonate
St-PEG	- polyethylene glycol stearate
TMA-PEG	- trimethylamino polyethylene glycol
wt/vol	- weight per volume
wt/wt	- weight per weight

Introduction

The attachment of bacteria to surfaces is a general phenomenon in natural environments. The importance of bacterial adsorption for the colonization of surfaces was early recognized by marine microbiologists and the mechanism by which marine bacteria attach to surfaces has attracted considerable interest (25, 98). It was early demonstrated that there is a selectivity of bacterial adsorption to inorganic solids (174). More recent studies by Gibbons and his colleagues have shown that there is also a selectivity of bacterial attachment to mammalian cells (41) and that this selectivity is the basis for tissue tropism. These studies have lead to an increasing interest in recent years in the factors determining bacterial adsorption. Adherence of bacteria is now considered to be the first step in the infectious process and is then followed by colonization and invasion.

The interaction between bacteria and mammalian cells involves non-specific factors such as charge and surface hydrophobicity and specific factors such as adhesins on the bacterial surface and complementary receptor structures on the host cell (9, 115). It has been shown by the work of Smith (133) that the physicochemical surface properties of bacteria are crucial for the outcome of a host-parasite interaction. Most mammalian cells as well as bacteria carry a net negative charge which creates repulsive forces between the cells. Several studies have indicated that hydrophobicity of microorganisms promotes the adherence to host cells (27, 68, 93, 119, 135, 137, 138, 154,157). The liability to hydrophobic interactions might serve to overcome the electrical

repulsion and thereby facilitate the interaction between specific binding molecules on bacteria and host cells. Studies on Salmonella typhi-murium have revealed that phagocytosis-sensitive R mutants are liable to hydrophobic and ionic interactions, whereas the smooth phagocytosis-resistant strains have a hydrophilic and uncharged surface (95, 136). These studies indicate a complexity of host-parasite interactions where the bacterial adhesiveness is not always beneficial to the bacteria.

Several methods have been described for measuring surface properties of bacteria (c.f. ref 92). In order to measure hydrophobic properties of particles such as bacteria, charge effects should be minimized (147). This can be achieved by lowering the pH, increasing the ionic strength, and using a buffer system with high salting-out effect. The different methods described for determining surface hydrophobicity include contact angle measurements of dried cell layers (154, 155), determination of the binding of hydrophobic probes to bacteria (64, 69, 96), hydrophobic interaction chromatography (53, 135), precipitation of cells by salts (91), adherence of bacteria to hydrocarbons (126), and partition of cells in aqueous polymer two-phase systems (2).

β -hemolytic streptococci

β -hemolytic streptococci are characterized by their ability to grow in chains and to produce zones of hemolysis around the colonies on blood agar plates. The β -hemolytic streptococci have by the work of Lancefield been separated into well defined serologic groups designated by the letters A through O (82). This division is based on the presence of group-specific carbohydrate antigens in the bacterial cell walls. Most strains associated with human infection belong to the species Streptococcus pyogenes, which constitutes the antigenic group A. A wide variety of clinical

manifestations are related to group A streptococcal infections. Group A streptococci are responsible not only for the acute streptococcal pharyngitis and its suppurative complications but also for nonsuppurative sequelae such as acute glomerulonephritis and rheumatic fever.

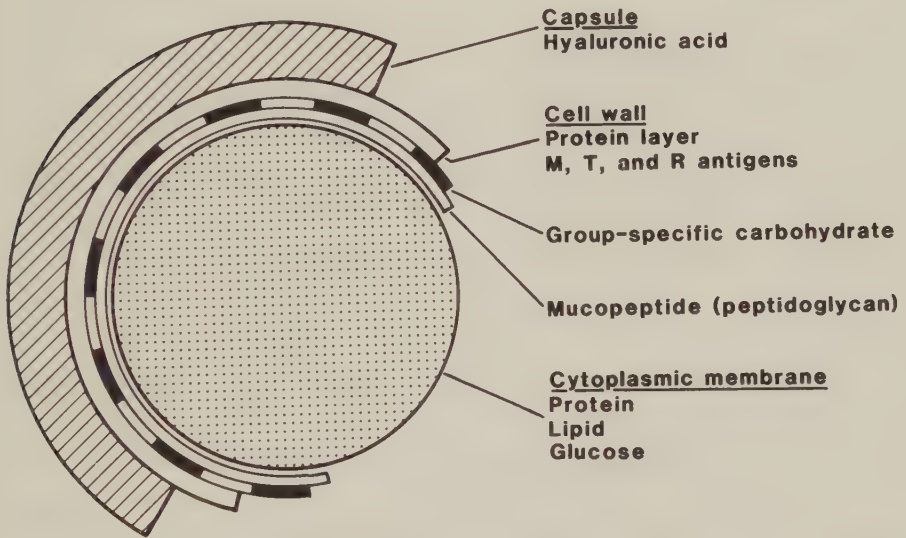


Figure 1. Diagrammatic representation of a group A streptococcal cell. Modified from Krause, R.M. (72).

A schematic diagram of the group A hemolytic streptococcal cell is shown in figure 1 (72). The cell wall of gram-positive bacteria is composed of peptidoglycan (129). Peptidoglycan is a polymer of N-acetyl-glucosamine and N-acetylmuramic acid to which a tetrapeptide is attached. The cell envelope of most gram-positive bacteria also contains other polymers such as teichoic acids, which are covalently linked to the peptidoglycan (6, 7, 161) and lipoteichoic acid also referred to as membrane teichoic acid (168).

Many strains of hemolytic streptococci produce capsules. The capsular substance of group A and C streptococci consists of hyaluronic acid. It is not immunogenic, probably because it is chemically indistinguishable from the ground substance of mammalian connective tissue. The role of the hyaluronic acid capsule in virulence is not clear but it may influence the resistance of the organisms to phagocytosis (52, 65, 140, 164).

1. Lipoteichoic acid (LTA)

All gram-positive cocci appear to have lipoteichoic acid in their cell wall (70). LTA is an amphiphatic molecule composed of 1,3-phosphodiester-linked glycerophosphate and a small lipid moiety (70, 168). It has generally been considered to be a membrane associated substance but can also be detected on the surface of intact bacteria and is excreted into the surrounding medium (4, 12, 62, 67, 97, 100, 153). Wicken and Knox have proposed that LTA may be transient within the cell wall (168). Extracellular LTA can exist as acylated micellar LTA and deacylated LTA (168). The hydrophobic lipid portion of acylated LTA is essential for most of the biological activities attributed to LTA (169). These include immunogenicity (167), binding to mammalian cell membranes (8, 10, 11, 114, 116), activation of the alternate complement pathway (34), hypersensitivity reactions and suppression of the immune response in laboratory animals (57, 103), cytotoxicity and inhibition of normal collagen synthesis in mouse fibroblasts (90), and stimulation of bone resorption in organ culture (48). LTA has also been suggested to play a central role in the pathogenesis of streptococcal infections (168). Beachey et al have suggested that LTA mediates the adhesion of streptococci to epithelial cells and to phagocytes (8, 12, 114, 116). Recently, Carruthers et al presented data indicating that LTA mediates the adherence of Staphylococcus aureus to buccal cells (20). LTA from cariogenic streptococci has also been suggested to play a role in the

attachment of these organisms to tooth surfaces (123, 124). Elevated levels of cellular LTAs of group B streptococci have been reported to be associated with human neonatal disease (112).

The topological localization of LTA has been a matter of controversy. The association of LTA with the cytoplasmatic membrane is well established (70) and it is presumed that the glycolipid portion of LTA interacts hydrophobically with the bilayer of the plasma membrane (70, 168). This orientation of LTA creates an apparant dilemma since the hydrophobic lipid portion is essential for most of the biological activities of the molecule. Recently Ofek et al , however, proposed that LTA is capable of forming a complex with M protein in such a way that the lipid moieties are free to interact with fatty acids (117).

2. Protein antigens

Three major protein antigens, designated M, T and R antigen, are present on the surface of group A streptococci (83). In addition, a non type-specific antigen closely related to M protein has been demonstrated in group A, C, and G streptococci (15, 156, 170). It has been proposed that these non-specific antigens should be referred to as M-associated proteins (MAP).

The most important factor influencing the virulence of group A streptococci is the M protein surface antigen (39, 87, 149). M proteins are antiphagocytic molecules that form a "fuzzy coat" of fimbriae on the surface of group A streptococci (142). Protective immunity to group A streptococcal infections is mediated by opsonic antibodies directed against the M antigen (86). More than 60 different M serotypes have been discovered (39). The recognition of M antigens and antibodies has special significance for epidemiological studies. Several methods have been described for assay of M antigens and antibodies (cf. ref. 39). The method

generally used for M-typing is the capillary precipitin test (144). The precipitin reaction is performed with acid extracted M protein and absorbed antisera. The M specificity can also be studied in complex biologic tests such as the mouse protection test (87) and bactericidal test (84, 99). In the mouse protection test, mice are injected intraperitoneally with antiserum and then challenged with a virulent streptococcus. The bactericidal test is frequently used to detect antibodies to M protein in human sera. It exploits the fact that M protein renders the streptococci resistant to phagocytosis by human leukocytes and that antibodies to M proteins are capable of neutralizing this antiphagocytic activity. The "long-chain" test for assay of type-specific antibodies is based on the observation that group A streptococci grown in the presence of homologous anti-M antibody creates elongated chains (139).

The conventional method of extracting M protein from group A streptococci is with hot dilute acid (81). Other methods described for extraction of M protein from whole streptococci include pepsin digestion (26), streptococcal phage lysine (37), alkaline extraction (40), and detergent treatment (35, 36). The lysine extracted molecule has nearly the same length as a fimbria, indicating that it represents most of the M molecule protruding from the surface (122). Pepsin and detergent extracted M protein represents, however, fragments from the distal part of the M molecule (122). Data have been presented indicating that the type-specific moiety of M protein differs from the antiphagocytic moiety of the molecule (36, 47). Beachey and Ofek have shown that streptococci lose their antiphagocytic properties after pepsin treatment (12) and that peptic fragments induce type-specific, protective antibodies indicating that both type-specific and antiphagocytic moiety of the M molecule resides in the distal pepsin fragment of M protein.

It has been demonstrated by Kantor that M protein will interact with fibrinogen to form insoluble complexes (63). The binding of aggregated β_2 -microglobulin to group A streptococci is closely related to the fibrinogen binding (76). M-positive strains of group A streptococci were found to bind β_2 m whereas the M negative variants were much less reactive (17). These data suggest that the receptor for aggregated β_2 m is located in close proximity to the fibrinogen receptor on M protein. Wittner and Fox found that traces of polyglycerophosphate will copurify with M protein (173) and that it could only be released from M protein by the action of enzymes. More recently, Ofek et al have suggested the formation of molecular complexes between M protein and the polyglycerophosphate backbone of lipoteichoic acid (117).

It has been claimed that the hydrophobicity of group A streptococci is related to M protein (151, 152, 158) and that M protein mediates the adherence of these organisms to epithelial cells (28). Other studies indicate, however, that there are no differences in adherence between M protein positive and M protein negative strains of streptococci (5). Recently Ofek et al presented evidence that the hydrophobic interaction between group A streptococci and hexadecan droplets is most likely due to glycolipids complexed with a surface protein (118). Several studies have further indicated that LTA rather than M protein is the adhesin mediating the binding of streptococci to epithelial cells (8, 9, 12).

The biological significance of T and R antigens is not known but neither seems to influence virulence. Two immunological distinct R antigens have been described and are designated 3R and 28R (85, 88). Group A streptococci can be subdivided into different T-types (44). T-typing is helpful in epidemiological studies since the different T-types are associated with a limited number of M anti-

gens and may thereby simplify the definite subdivision of group A streptococci depending on the type of M antigen.

3. Extracellular products

A wide variety of extracellular substances are produced by group A streptococci. The results of serological and electrophoretic studies indicate that as many as 20 different extracellular antigens may be released by group A streptococci (45). Some of these substances have been studied extensively. Many of the clinical manifestations of streptococcal infections are related to extracellular products. The most important extracellular products are summarized in Table 1.

Table 1. Extracellular products of group A streptococci

Erythrogenic toxins (A, B and C)	Causes rash of scharlet fever
Streptolysin S	Produces hemolysis on blood agar plates. Stable in air. Does not stimulate production of inhibitory antibody.
Streptolysin O	Produces hemolysis on blood agar plates. Oxygen labil. Causes lysis of cytoplasmatic granules.
Diphosphopyridine nucleotidase	Splits NAD
Deoxyribonuclease	Four different types; A though D. Depolymerizes DNA.
Streptokinase (A and B)	Catalyze the conversion of plasminogen to plasmin which digests fibrin.
Hyaluronidase	Depolymerizes hyaluronic acid
Proteinase	Hydrolyzes M protein.

4. Bacterial receptors for human proteins

Recently, an increasing number of specific interactions between surface structures of gram-positive cocci and human proteins have been discovered (cf.ref.75). Immunoglobulin (Ig) Fc binding properties of gram-positive cocci were first discovered in staphylococci (38) but are now known also to reside in group A, C and G streptococci (21, 74). Five major types of Fc-receptors that differ in their specificity have been discovered. Fc-receptor type I is represented by staphylococcal protein A; type II-receptors are found in group A streptococci; type III-receptors are represented by human group C and G streptococci; type IV-receptors are found in bovine group G streptococcal strains; and type V-receptors are confined to the group C species Streptococcus zoo-epidemicus (105, 106, 108). An alternative nonimmune Ig binding mediated by the Fab-regions has been demonstrated for staphylococcal protein A and streptococci (29, 32, 56). An Fc-specific IgA receptor binding both IgA subclasses have been detected in some group A streptococci (22, 128).

Apart from the described non-immune Ig binding there are five defined types of specific interactions between streptococci and human proteins: (i) Fibrinogen binds to group A, C and G streptococci as well as to Staphylococcus aureus (77, 148). In the case of group A streptococci the fibrinogen receptor has been correlated to M protein and is probably an integral part of this protein (63). (ii) Haptoglobulin shows affinity for some group A streptococci and binds to T4 associated structures (71). (iii) Recently, specific binding sites for human serum albumin has been demonstrated in group A, C and G streptococci (78, 107, 171). Five types of albumin-receptors different from other known human serum-protein receptors have been defined (171, 172). (iv) The binding of fibronectin to bacteria was first discovered in protein A carrying staphylococci (80). More recent studies have demonstrated

a binding of fibronectin to group A, C and G streptococci (13, 109, 132, 145). (v) The close relationship between β_2m and immunoglobulins (120, 134) raised the question whether β_2m could react with Fc-reactive structures on bacteria. It was found that aggregated β_2m bound to surface structures on group A, B, C and G streptococci (76, 130). The β_2m -binding structure was, however, different from the Ig Fc receptor (76). The receptor for aggregate β_2m seems to be located on M protein in group A streptococci (17, 159). β_2m is a low-molecular-weight protein present not only in plasma and urine but also in other body fluids and on the surface of all nucleated cells (16, 113, 120). At the cell surface β_2m represents the invariant light chain of the major histocompatibility complex (MHC) class I antigens (43, 111, 121). This localisation suggest that β_2m could promote the adherence of group A streptococci to epithelial cells (18).

Materials and Methods

1. Bacterial strains, cultivation

The bacterial strains used in this study included collections of strains obtained from other laboratories or strains obtained from clinical specimens sent to the Clinical Microbiology Laboratory, University Hospital, Lund, Sweden. Strains were stored at -70°C in Todd-Hewitt broth supplemented with fetal calf serum or on blood agar plates at 4°C . Todd-Hewitt broth or tryptone broth was inoculated and incubated overnight at 37°C . Bacteria were harvested by centrifugation at 3000 rpm for 15 minutes and washed twice in PBS. The optical density at 620 nm was measured and the bacterial concentration was calculated from a standard curve and adjusted to 10^9 organisms per ml. In some experiments the bacteria were submitted to heat treatment at 80°C for 5 minutes.

2. Radiolabelling of bacteria

Radiolabelling of bacteria was performed with four different radioactive isotopes. Labelling with ^{125}I was performed by suspending 10^9 bacteria in 200 μl of PBS. Then 15 μCi of ^{125}I , 5 μl of 0.015 % hydrogen peroxide in PBS, and 5 μl lactoperoxidase were added to the cell suspension and incubated at room temperature for 10 min. Internal labelling with [^3H]thymidine was performed by growing the bacteria in Todd-Hewitt broth containing 2 μCi of [^3H]thymidine per ml. Bacteria were labelled with ^{51}Cr by adding 50 μCi of the isotope to 10^9 bacteria in PBS and incubating the mixture at 37°C for 3 h. LTA was radiolabelled by growing the bacteria in Todd-Hewitt broth supplemented with 1.5 μCi of [2- ^3H]

glycerol per ml. After the different labelling procedures, the bacteria were washed four to five times in PBS and suspended in appropriate solutions.

3. Radiolabelling of serum proteins

Human serum albumin, IgG, IgA, and β_2 -microglobulin were labelled with ^{125}I using the chloramine-T method (55, 101). Fibrinogen was radiolabelled by using the electrolytic method of Rosa et al (125) as described by Harboe and Fölling (46).

4. β_2 -microglobulin liposomes

β_{2m} is not an integral membrane protein and is not incorporated into the lipid bilayer of conventional liposomes. Instead, β_{2m} was covalently linked to aldehyde groups generated on the outer surface of liposomes prepared from a mixture of phosphatidylcholin, phosphatidylinositol, cholesterol and a semi-synthetic glycolipid (49, 141, 146). The β_{2m} liposomes were freed from non-coupled protein by flotation through dextran T40. A high recovery of liposomes was obtained (> 90 per cent) but the yield of coupled protein was low (< 5 per cent). The density of β_{2m} molecules attached to the liposomes was equivalent to the density of β_{2m} on human lymphocytes.

5. Human protein binding assay

Interactions between human proteins and bacteria was studied in a direct binding assay employing radiolabelled proteins. A 200 μl suspension of 2×10^8 bacteria in PBS with 0.05 % Tween 20 was added to radiolabelled proteins. After 1 h at room temperature, 2 ml of PBS with 0.05 % Tween 20 was added to each tube and the bacteria were deposited by centrifugation. Supernatants were discarded and the radioactivity in the bacterial pellets was measured.

6. Polymer two-phase systems

Polyethylene glycol (PEG) 4000 and PEG 6000 separating into the upper phase and dextran T500 separating into the lower phase were used for polymer phase systems as described by Albertsson (1, 2). Two-phase systems were prepared from stock solutions of PEG (40%) and dextran (20%). Charged polymers for cross-partition experiments were maintained in 10 % stock solutions whereas the hydrophobic PEG esters used in hydrophobic affinity partition experiments were 1.0 to 5.0 %. Salt and buffer stock solutions were made up to concentrations 10 to 20 times higher than that of the final concentration in the phase system. Initially, the polymer solutions were weighed in each tube, and appropriate buffer and salt solutions were then added with a pipette. In further experiments, batch systems were prepared from stock solutions of PEG, dextran, salt and buffer. The batch system was mixed thoroughly and multiple samples were taken using a Cornwall pipette. After addition of charged or esterified PEG and radiolabelled bacteria the tubes were inverted about 50 times. After 40-min settling time 100 μ l of the top and bottom phases was withdrawn from the tubes and the radioactivity in each sample was measured in a gamma counter or beta scintillation counter. All phase systems were prepared and all experiments were carried out at room temperature.

6.1 *Hydrophobic affinity partition*

The hydrophobic surface properties of bacteria were studied by hydrophobic affinity partition in two-phase systems containing PEG palmitate (P-PEG) or PEG stearate (St-PEG) (2, 31, 160, 163). The PEG esters (C_8 to C_{18} fatty acid esters of PEG 6000) used in some experiments were prepared with dicyclohexylcarbodiimide mainly according to Johansson (58). The composition of the phase systems used were as follows: 6.1 % (wt/wt) dextran, 6.1% (wt/wt) PEG 4000, 0 to 0.5% (wt/wt) PEG esters, 5mM sodium phosphate buffer, and 40 mM NaCl.

6.2 Cross-partition

Single tube cross-partition experiments were performed in order to estimate the isoelectric point of bacteria (3, 30, 54). Two series of phase systems were prepared, one with positively charged TMA-PEG and one with negatively charged S-PEG. The composition of the phase systems were as follows: 6.8% (wt/wt) dextran, 4.8% (wt/wt) PEG 4000, 2.0% (wt/wt) charged PEG, and 2.5 mM buffer.

6.3 Presentation of partitioning data

In phase systems selected to study the effect of protein binding on the surface properties of bacteria the organisms partitioned mainly into the upper phase and interface in the absence of sodium chloride. The addition of increasing amounts of NaCl changed the partition of the bacteria in favour of the lower phase and the results are presented as per cent bacterial cells in the lower dextran rich phase. Differences in partition behaviour between bacteria pretreated with serum proteins and untreated bacteria were indicative of changed physicochemical surface properties.

Radiolabelled proteins partition between the upper phase and the lower phase in polymer two-phase systems. The results were presented as partition coefficient (K) defined as concentration in top phase divided by concentration in bottom phase.

The results from cross-partition experiments were presented as the cross-point between the two curves obtained when the percentage of bacteria in the top phase is plotted against the pH. Similar results were obtained when samples were taken from the bottom phases. The obtained cross-point is taken as the approximate isoelectric point of the bacteria (3).

Surface hydrophobicity of bacteria was assessed by determining the

affinity of the bacteria for PEG-esters which results in a transfer of bacteria in the direction of the top phase. In the initial experiments a hydrophobicity index (C) was calculated for comparison of the relative hydrophobicities among the various bacterial strains. C was calculated from the following formula: $C = \frac{\text{counts per minute in bottom phase in system with esterified PEG}}{\text{counts per minute in bottom phase in system without hydrophobic groups}}$. The value of C is below 1 when a hydrophobic interaction takes place. A C value above 1 means that the cells are excluded from the upper phase and/or interface when the fatty acid group is introduced. In further hydrophobic affinity experiments the partitioning data were presented as change of partition (CP) as described by Magnusson et al (94). This method has the advantage that it takes into account all transfers of bacteria between top and bottom phase and the interface of a two-phase system.

7. Enzymatic treatment of group A streptococci

The differential effect of proteolytic enzymes on bacterial surface structures was utilized to characterize the hydrophobic factor on the surface of group A streptococci. Enzymatic digestion was performed by suspending 5.0×10^8 bacteria in 0.5 ml PBS containing various amounts of trypsin, pepsin, papain or hyaluronidase. The mixtures were incubated 30 min (pepsin), 45 min (trypsin and papain) or 60 min (hyaluronidase) at 37°C . The trypsin digestion was terminated by addition of 0.5 mg trypsin inhibitor and the pepsin activity by addition of 100 μl of 1M Tris base. After enzymatic treatment, the bacteria were washed twice in PBS and suspended in appropriate solutions.

8. Extraction of LTA

The extraction of LTA from group A streptococci was performed with hot phenol by the method of Westphal and Luderitz (162) as

described by Wicken et al (166). A bacterial pellet was suspended in distilled water and an equal volume 90 % (wt/vol) phenol at 65°C. After mixing for 5 min, the suspension was centrifuged and the aqueous phase removed. The extraction was repeated once and the combined extracts were dialyzed against 6 changes of distilled water.

9. LTA-assays

A novel method for quantitation of cell surface LTA was developed and is presented in the Results section. Three methods were used to demonstrate and quantitate LTA in extracts and culture fluids. The antiserum against LTA used in the different serological tests was a kind gift from Dr, K.W. Knox, Sydney, Australia. This rabbit antiserum was raised against chloroform-water extracted L. casei 6375-LTA. The specificity of the antibodies is towards the polyglycerophosphate backbone of LTA (166). The presence of radio-labelled LTA in extracts was demonstrated by precipitation with an antiserum against L. casei LTA. Fifty µl of LTA antiserum and 25 µl of tritiated aqueous phenol extract was mixed and diluted with 175 µl PBS. Normal rabbit serum was used as a control. After incubation overnight at 37°C, 150 µl of bovine serum and 800 µl of 15 % PEG 6000 were added to each tube. The mixture was centrifuged at 1,000xg for 45 min. Supernatant was discarded and the radioactivity in the pellet was measured in a beta scintillation counter.

The RBC-sensitizing activity of LTA was assayed essentially as described by Ofek et al (116). Briefly, papain-treated 2 % RBC were added to serial two-fold dilutions of LTA. After incubation at 37°C for 45 min the erythrocytes were washed twice in PBS and suspended in anti-LTA diluted 1/100 in PBS. The mixture was incubated for 30 min at 37°C and centrifuged 5 min at 1,000xg. The reciprocal of the highest dilution which sensitized erythrocytes

fully was designated the LTA sensitizing activity. The formation of extracellular LTA was assayed by the same method using cell free neutralized culture fluids (97).

Rocket immunoelectrophoresis (89) which will measure both acylated and deacylated LTA was performed essentially as described by Wicken et al (165). The electrophoresis was performed in a 0.8 % agarose gel in veronal Ca^{++} buffer containing 2 % antiserum to L. casei LTA.

10. RIA utilizing staphylococcal protein A as an anti-antibody reagent

The use of staphylococcal protein A as an immunochemical reagent relies on the specific binding of protein A to the Fc region of IgG (38, 42). Protein A was labelled with ^{125}I by the chloramin-T method (55) and used as a second layer anti-antibody in the assays of type-specific M antigens and LTA on whole group A streptococci.

Aims of the Investigation

The present investigations were intended

- to study the effect of specific binding of human serum proteins to streptococci and staphylococci on surface properties of the bacteria
- to study surface properties of streptococci and staphylococci with emphasis on surface charge and hydrophobicity
- to characterize the surface structure on group A streptococci responsible for surface hydrophobicity
- to determine the role of M protein in surface hydrophobicity of group A streptococci
- to further analyze the interaction between β_2 -microglobulin and group A streptococci

Results and Discussion

1. Polymer two-phase systems (I, II, III, IV)

1.1 *Selection of two-phase systems*

The selection of a suitable phase system was performed as outlined by Albertsson (2). Factors affecting the partition of particles such as bacteria include polymer concentration and salt composition. In the initial studies on the effect of protein binding on partition of the bacteria a phase system was sought for each bacterial strain in which the bacteria partitioned mainly into the upper phase and interface. Phase system A and B (Table II) were selected for investigations on the effect of protein binding on the surface properties of bacteria. The addition of increasing amounts of sodium chloride to these phase systems will change the partition of the bacteria in favour of the bottom phase (2).

The polymer concentration chosen for cross-partition experiments was much above the critical composition, that is the polymer concentrations below which two-phase systems are no longer obtained (Phase systems C and D, Table 2). The higher polymer concentration will result in a more even distribution of the bacteria between the phases. Two series of phase systems were prepared, one with positively charged TMA-PEG and one with negatively charged S-PEG. By addition of buffer, varying pH was obtained in the phase systems. The partition of the bacteria was then studied as a function of pH with the two different charged PEG-molecules.

Table 2. Composition of phase systems for single-tube partition

Phase system	% (wt/wt)					m M				
	Dextran T500	PEG 4000	PEG 6000	TMA-PEG	S-PEG esters	Buffer (see text)	Sodium phosphate buffer pH 7.8	Sodium phosphate buffer pH 6.9	Lithium phosphate buffer pH 6.8	NaCl
A	5.0		4.0				10.0			0-40.0
B	5.0		4.0						10.0	0-40.0
C	6.8	4.8		2.0		2.5				
D	6.8	4.8			2.0	2.5				
E	6.1	6.1			0-0.5			5.0		40.0

The addition of certain mixtures of salts, for example sodium chloride and sodium phosphate, will create a phase system with zero interfacial potential (2). In phase systems with this salt composition the bacteria will distribute according to the ratio between hydrophobic and hydrophilic sites exposed on their surfaces. This phase system was therefore chosen for hydrophobic affinity partition experiments (Phase system E, Table 2). Most of the bacteria partitioned in this phase system into the bottom phase in the absence of esterified PEG. The addition of hydrophobic PEG-esters resulted in a transfer of the bacteria from the bottom phase mainly into the interface.

2. Effect of different labelling procedures on the partition of the bacteria (III)

The isotopes [^3H]thymidine, ^{125}I and ^{51}Cr were used for bacterial labelling. The influence of the different labelling procedures on the hydrophobic affinity partition was investigated. Bacteria labelled with the three different radioisotopes behaved similarly in hydrophobic affinity partition, indicating that no major changes occurred due to radiolabelling. For routine labelling gamma emitters were preferred to beta emitters since the samples are easier to count and pose fewer handling problems. ^{125}I was found most suitable among the gamma emitters since the labelling procedure is less time consuming than for ^{51}Cr (10 min as compared to 3h) and ^{125}I has a longer half-life.

3. Effect of specific binding of human proteins on the surface properties of bacteria (I,II, IV)

3.1 *Distribution of bacteria in phase systems after specific protein binding* (I)

Five strains with different combinations of positive binding of human proteins were selected for partition studies and included

one S. aureus strain, two group A streptococcal strains and one group G streptococcal strain. The effect of serum, human serum albumin, IgG myeloma protein, and human fibrinogen on the partition of the bacteria was investigated. The partition of all four strains studied was strongly influenced by the addition of human serum and from experiments with varying dilutions of serum it was shown that the change of partition was dose-dependent. The effect of human serum albumin, IgG myeloma protein and fibrinogen on the partition of the bacteria correlated to the binding of radiolabelled proteins to the bacterial strains (Table 3). As for human albumin and IgG the effect on the partition of the bacteria was shown to be dose-dependent. The results demonstrated that the specific binding of proteins to the bacteria changes their physico-chemical surface properties.

Table 3. Effect of binding of human proteins to bacterial strains on the partition in two-phase systems in relation to binding of radiolabelled human proteins to the same strains.

Bacterial strains	Serum	Human albumin	IgG	Fibrinogen
<u>S. aureus</u>	++	- (2)	+++ (77)	+ (16)
Group A streptococcus, AW43	++	+ (5)	++ (26)	++ (53)
Group A streptococcus, AM1	++	- (8)	++ (67)	+ (57)
Group G streptococcus, G148	+++	+++ (70)	++ (75)	- (7)

One, two, and three arrows, respectively, denote low, medium and high effect of protein on partition of bacteria in the two-phase systems. A dash represents no effect. The direction of the arrow denotes a change of partition in favour of the top phase (↑) or the bottom phase (↓) in the presence of protein as compared to the partition of bacteria alone. Figures within brackets denote percent uptake of radiolabelled proteins.

There are several possible mechanisms by which a specific protein-binding could change the surface properties of a bacteria. The change in distribution of the bacteria after protein binding might be ascribed to the partition properties of the bound protein itself. However, studies on the partition of radiolabelled protein alone indicated that the partition properties of bound protein could not be the sole factor influencing the distribution of the bacteria since one protein could have adverse effects on the partition of different bacterial strains (Table 3).

It could be possible though, that a protein bound to the bacterial surface exerts different physico-chemical properties as compared to the same protein in solution. As for gammaglobulin the non-immune Fc-binding will orientate the molecules with their more hydrophilic Fab parts outward. A third mechanism by which a protein binding could lead to changes in surface properties is through the masking of bacterial cell surface properties. Bound proteins might cover receptor areas that are no longer available to the polymers. This possible mechanism is supported not only by the adverse effects of individual proteins on the partition of the different strains but also by the similarity in effect of different proteins on the partition of the individual bacterial strains (i.e. the direction of change in partition; Table 3)

Phase partitioning was found to be a sensitive tool for the study of surface properties of bacteria and their changes after interaction with proteins. This method could be of value for discovering unrecognized specific protein binding to bacterial cells.

3.2 *Effect of human serum on isoelectric points and hydrophobic affinity of bacteria (II, IV)*

The effect of human serum on the isoelectric point and hydrophobic interaction liability was studied in polymer two-phase systems. A 100 μ l portion of normal human serum, diluted 1:10 in PBS, was added to approximately 1×10^9 bacteria and incubated at room temperature for 30 min. The bacteria were then washed twice in PBS before use in two-phase systems. Cross-partition was performed on four streptococcal strains and one staphylococcal strain with and without pretreatment with human serum. The cross-partition of all four streptococcal strains was strongly influenced by pretreatment with human serum, resulting in less acidic cross-points. The pretreatment of the staphylococcal strain with human serum did however not lead to any detectable change of isoelectric point by this method.

Surface hydrophobicity of eight group A streptococcal strains was determined in hydrophobic affinity partition after pretreatment with human serum and was compared to the hydrophobicity of the same bacteria pretreated with PBS alone. The hydrophobic affinity was in all instances higher after pretreatment with human serum. The effect was more pronounced with bacteria having a hydrophobic surface prior to pretreatment with human serum. All bacteria used in these studies carry specific receptors for human serum proteins and the effect of human serum on the isoelectric point and hydrophobic affinity of the bacteria is most likely due to specific binding of proteins to their surfaces. The pretreatment of bacteria with human serum was performed in PBS without Tween since this detergent could influence the hydrophobic affinity partition. This means that low avid non-specific interactions between serum proteins and the bacteria also might have contributed to the noted effects.

3.3 *Biological significance of protein binding to bacteria*

The major types of bacteria-protein interactions have been reviewed in the introduction. Not only bacteria but also other microorganisms such as Schistosoma mansoni are capable of binding human proteins to their surfaces. This mammalian parasite is capable of specific binding of blood group substances and histocompatibility antigens to its surface (23, 131). Receptors for IgG Fc and human β_2 -microglobulin have also been discovered on the surface of S. mansoni (66, 150). The occurrence of specific protein receptors in such widely separated species as schistosomes and streptococci indicate that protein binding might be a more general phenomenon among microorganisms. The biological significance of specific protein binding is not clear. The theme of host mimicry could offer a possible explanation for the role of specific protein binding to microorganisms (19). Our results demonstrate that a protein binding also could influence the host-parasite interaction on a physico-chemical level. It is difficult to predict the effect of changed surface properties of the bacteria on the outcome of a host-parasite interaction since the changes in surface properties might have adverse effects in the different stages of the infection process. The increased hydrophobicity and less acidic isoelectric point of streptococci after pretreatment with human serum might promote adherence to oropharyngeal cells but also facilitate the adherence to phagocytic cells and the subsequent elimination of the bacteria.

The protein binding structure on the surface of bacteria might also serve as an adhesin in the interaction with a complementary cell-bound protein receptor. This mechanism has been suggested for the adherence of group A streptococci to epithelial cells where M protein of group A streptococci interacts with cell-bound β_2 -microglobulin (18).

4. Surface properties of different gram-positive cocci (II)

4.1 *Isoelectric points of bacteria*

The isoelectric point of 22 streptococcal strains and 9 staphylococcal strains was determined by cross-partition. The results obtained indicated isoelectric points for streptococci around pH 3.75. The staphylococci showed an isoelectric point around pH 2 and differed thereby markedly from the streptococci. Freshly isolated strains did not differ significantly in isoelectric point as compared to laboratory maintained strains. The cross-points of heat-inactivated streptococcal strains (80°C, 5 min) were in all instances lower than the corresponding live bacteria whereas heat treatment of staphylococci resulted in a less acidic cross-point.

Cross-partition has previously been used for determination of the isoelectric points of proteins, mitochondrial membranes, spinach thylakoid membranes and rat liver peroxisomes (3, 30, 54). Investigations on proteins and rat liver mitochondria have shown a good agreement between cross-points and the isoelectric points as determined by isoelectric focusing (3, 30). The isoelectric point of bacteria has previously been estimated on group A streptococci. Electrokinetic studies on different strains of group A streptococci indicate an isoelectric point between pH 4.3 and 4.7 (51). Preliminary results from isoelectric focusing of M protein positive and M protein negative variants of group A streptococci suggest isoelectric points of pH 5.8 and 6.0, respectively (151). Several studies have indicated that surface charge could influence a host parasite interaction (110, 136, 138, 154). Our studies did not reveal any significant differences in isoelectric points between individual strains of group A, C and G streptococci indicating that all strains have a common basic surface structure. It also appears that the presence of M protein on group A strepto-

cocci does not lead to any major change in isoelectric point of the bacteria.

4.2 *Hydrophobic affinity of bacteria*

Relative surface hydrophobicity was determined on 39 streptococcal strains and 12 staphylococcal strains by hydrophobic affinity partition. This method is based on the assumption that PEG-esters included in the two-phase system will interact with all available hydrophobic sites on the bacterial surface and thus transfer the bacteria from the lower phase into the interface or upper phase (58). The streptococci varied greatly in their affinity for the PEG-bound fatty acids. Most group A streptococci showed a high hydrophobic affinity whereas the remaining four strains showed no affinity for the PEG-bound hydrophobic groups. Among the group C and G streptococci there was a more even distribution of strains with a high, medium, and low hydrophobic affinity. None of the investigated staphylococci showed any affinity for the PEG-bound hydrophobic groups.

Previous studies on Staphylococcus saprophyticus and Staphylococcus epidermidis have indicated a poor hydrophobic interaction liability of these bacteria (24). In our studies Staphylococcus aureus also showed a low hydrophobic affinity. This is in contrast to results obtained with hydrophobic interaction chromatography indicating a high surface hydrophobicity of S. aureus strains (61). As seen from cross-partition experiments the staphylococci have a highly negatively charged surface and this might prevent the interaction between hydrophobic molecules on the bacterial surface and the PEG esters. Further studies are needed to elucidate the relative hydrophobicity of S. aureus.

Varying degrees of hydrophobic affinity was noted among the streptococcal strains and it was at this stage tempting to speculate

that the observed differences were related to the occurrence of protein receptors on the surface of the bacteria. The binding of different radiolabelled human proteins was therefore determined on all strains included in the study. The presence of receptors for IgA, IgG, albumin and fibrinogen did not correlate to the hydrophobic affinity of the bacteria. In this primary study including a limited number of strains a correlation was found between the hydrophobic affinity of group A streptococci and the density of receptors for aggregated β_2 -microglobulin. More recent studies on a larger number of strains did, however, demonstrate that there is no correlation between the surface hydrophobicity of group A streptococci and β_2 m-binding. Since M protein appears to be the receptor for aggregated β_2 -microglobulin, our later results would indicate that there is no relationship between M protein and the hydrophobic properties of group A streptococci. This is in contrast to the report by Tylewska et al on the contribution of M protein to the hydrophobic surface properties of group A streptococci.

5. Surface hydrophobicity of group A streptococci (III, IV)

Previous results on the hydrophobic affinity of group A streptococci encouraged us to further investigate the hydrophobic structure on the surface of these bacteria. The differential effect of proteolytic enzymes on bacterial surface structures was utilized to characterize the hydrophobic factor on group A streptococci. Trypsin, papain, and pepsin (pH 4.5) treatment resulted in a marked decrease in hydrophobic affinity as measured in polymer two-phase systems. Mild pepsin digestion (pH 5.8) which is known to cleave the distal portion of the M molecule (122) had however no significant effect on the hydrophobic affinity partition of the bacteria. These results indicated that the hydrophobic structure on group A streptococci either consists of a surface protein other than M protein or that it consists of molecules bound to a pro-

tein. Our interest was then focused on lipoteichoic acid since it is known that streptococcal surface proteins solubilized by proteolytic digestion often contains LTA (60). Several reports also indicated that this cell surface polymer plays an important role in the pathogenesis of streptococcal infections (8, 12, 114, 116, 168).

The development of novel radioimmunoassays for surface LTA and M protein enabled us to investigate the effect of proteolytic digestion on these surface components. Enzymatic digestion with trypsin, papain and pepsin (pH 4.5) resulted in a removal of both surface LTA and the M antigen. Mild pepsin digestion (pH 5.8) also resulted in a loss of M antigen whereas the amount of surface LTA was unaffected. A direct comparison between surface hydrophobicity as measured in polymer two-phase systems and the amount of surface LTA was also made. The results showed a good correlation between the two parameters. Taken together this would indicate that LTA is the major hydrophobic constituent of group A streptococci. This was further supported by partition experiments performed with PEG-bound fatty acids of varying lengths. The selectivity of fatty acid esters with different chain lengths on the partition of chloroplasts has previously been demonstrated (59). The partition of whole group A streptococci revealed that a chain length of more than 13 carbon atoms was required for a hydrophobic interaction to take place. Radiolabelled solubilized LTA showed a similar partition behaviour indicating that LTA in situ is the major factor responsible for the hydrophobic interaction. Purified radiolabelled M protein (79) differed markedly from whole group A streptococci and solubilized LTA when submitted to partition in the same phase systems. A hydrophobic interaction with M protein was present already with C₉ fatty acid and the hydrophobic affinity was also much lower then for LTA (H. Miörner, O. Kühnemund, and G. Kronvall, unpublished).

Relative surface hydrophobicity and LTA content was also determined on a collection of M-positive and M-negative group A streptococcal reference strains. The hydrophobic affinity and amount of surface LTA varied greatly between individual strains. The obtained data were submitted to statistical evaluation using the Wilcoxon test (104). The differences in surface hydrophobicity between M-positive and M-negative strains were not significant whereas the differences in surface LTA content between the two groups was highly significant. However, looking at individual strains it was evident that bacteria with a high hydrophobic affinity and a high content of surface LTA were found among both M protein positive and M protein negative strains. In addition several M-positive strains showed a low hydrophobic interaction liability and a low surface LTA content. The nature of the surface protein to which LTA is bound is not clear. Ofek et al have suggested that LTA is anchored to M protein by its polyglycerophosphate backbone (117). Our results revealed, however, that LTA is not bound to a proximal part of the M protein which exerts any antiphagocytic or M type antigen properties. The possibility still remains that LTA is bound to a portion of M protein near the surface of the bacteria.

The great variation in hydrophobic affinity and surface LTA content among individual M-positive and M-negative strains indicated that surface hydrophobicity does not play a crucial role in the interaction of streptococci with phagocytes. Our results are consistent with the idea that different mechanisms are involved in adherence of bacteria to epithelial cells and to phagocytes (12, 73, 143).

6. Determination of surface LTA and M protein on whole bacterial cells . (III, IV, V, VI)

6.1 *LTA-assay* (III, IV, VI)

Various methods have been described for the assay of solubilized LTA (168). Lipoteichoic acids will sensitize erythrocytes and this has been utilized in a very sensitive hemagglutination assay (50). The RBC-sensitizing assay will measure only acylated LTA since the lipid portion is essential for the spontaneous binding to erythrocytes. Rocket immunoelectrophoresis measures both acylated and deacylated LTA (166). Several reports have indicated that LTA is present on the surface of gram-positive cocci (12, 97, 100, 153, 168) and this encouraged us to develop an assay for surface associated LTA on whole bacterial organisms.

A rabbit antiserum raised against Lactobacillus casei LTA was kindly provided by Dr. K.W. Knox, Sydney, Australia. These antibodies are specific for the glycerol phosphate sequence of LTA and are known to cross-react with a variety of lipoteichoic acids (70). Bacteria were incubated with anti-LTA and with normal rabbit serum as a control. The presence of antibodies specific for LTA on the surface of bacteria was demonstrated by addition of ^{125}I -labelled protein A (42). Gram-positive bacteria were found to have varying amounts of surface LTA whereas none of the gram-negative bacteria tested bound any anti-LTA. Inter- and intra-assay variation of the LTA assay indicated an acceptable discriminating power of the test. Polyclonal rabbit IgG is known to interact with Fc receptors on group A streptococci (106). This could theoretically create a dilemma but control experiments with normal rabbit serum indicated that the binding of rabbit IgG to Fc-receptors on group A streptococci prevented the subsequent binding of protein A to the Fc-fragment.

6.2 *M*-antigen assay (V, VI)

Several methods have been described for the assay of *M* antigen (cf.ref. 39). The typing of *M* antigen is usually done by the Lancefield capillary precipitin test (144) or by the Ouchterlony immunodiffusion assay (102, 127) using acid extracted *M* protein and specific rabbit antisera.

A radioimmunoassay for type-specific *M* antigens on whole group A streptococci was developed on the analogy of the above described LTA assay. Group A streptococci were incubated with rabbit anti-*M* antisera and in a second step with ^{125}I -labelled protein A. Initial experiments indicated the need for absorption of antisera. The removal of cross-reacting antibodies is a frequently encountered problem with antisera prepared against whole heat-killed cells. The absorption often leads to a considerable loss of type-specific antibodies. This represented however no problem when the antisera were absorbed with a heterologous streptococcal strain for use in the described RIA. Absorbed antisera directed against *M* types 12 and 49 were tested against 10 homologous, 48 heterologous, and 8 *M* negative strains. Both antisera gave a unequivocal reaction with homologous serotypes distinct from the weaker reaction with heterologous serotypes. The type-specificity of the reaction was further confirmed by removal of type-specific antibodies after absorption of a M12 antiserum with purified M12 protein coupled to Sepharose 4B.

It was surprising that one absorption with a heterologous group A streptococcal strain was sufficient to remove major cross-reacting antibodies without any appreciable loss of type-specific antibodies. The nonimmun binding of IgG to Fc receptors on group A streptococci could create a problem when absorbing antisera.

Kronvall et al (78) have, however, estimated that the nonimmune Ig-binding in an absorption procedure as the one used in this assay would only reduce the serum IgG concentration by about fifteen per cent. These estimations are compatible with the results obtained in our assay.

The described assay for type-specific M-antigens has the advantage that a simple absorption and dilution of antisera is sufficient to obtain a type-specific reagent for use in the radioimmuno-assay. With this method one can also circumvent the time consuming M protein extraction procedure. Preliminary results have indicated the possibility to perform the test on bacteria taken directly from a blood agar plate and suspended in PBS. This would further simplify the typing procedure. Studies on the effect of proteolytic digestion on M protein of group A streptococci have shown that the described M assay offers a valuable tool for semiquantitative estimation of M antigen in situ.

7. Interaction between β_2 -microglobulin and group A streptococci (VI)

Aggregated β_2^m is known to bind to surface structures on group A B, C, and G streptococci (76, 130). It has been suggested that the binding of β_2^m to group A streptococci is mediated by M protein (17, 159) and our aim was to further analyze this interaction.

A total of 45 M protein positive and 7 M protein negative group A streptococcal strains were tested in a direct binding assay employing radiolabelled aggregated β_2^m . The results indicated a large variation in binding capacity among the various M-positive strains whereas none of the M-negative strains bound β_2^m . The binding capacity among group A streptococci of the same M type did however not vary. Thus, fortysix M-type 12 and fourteen M-type 49 strains originating from different outbreaks of group A strepto-

coccal infections were all highly β_2m -reactive.

In an attempt to mimic the in vivo situation β_2m was covalently bound to a liposome. These liposomes were used in binding experiments and the results were compared to the capacity of the same strains to bind aggregated β_2m . β_2m liposomes were found to have affinity for M protein positive strains whereas the M protein negative strains were less reactive. The binding of radiolabelled liposomes could be blocked by addition of nonlabelled β_2m liposomes and by nonlabelled aggregated β_2m . The results indicated that β_2m aggregates and liposomes interact with the streptococcal surface in a similar way and justifies the use of β_2m aggregates in our routine binding assay.

β_2m is expressed at the surface of all nucleated cells and is non-covalently associated with HLA-antigens (16, 113, 120). The question arose whether β_2m associated to HLA-antigens still retains the capacity to bind to group A streptococci. This was investigated in binding studies employing monomeric and aggregated HLA-antigens. Monomeric HLA-antigens did not show any significant binding to group A streptococci whereas aggregated HLA-antigens showed a very similar binding pattern compared to aggregated β_2m . The binding of HLA aggregates was completely inhibited by aggregated β_2m . The results demonstrate that β_2m also in association with the heavy HLA chain have affinity for streptococcal surface structures.

It has been suggested that LTA forms a complex with M protein (117). It was therefore of interest to study the role of LTA in the interaction between M protein and aggregated β_2m . A direct comparison between surface LTA content and uptake of aggregated β_2m on 28 group A streptococcal strains showed no correlation. It was further shown that pretreatment of bacteria with sodium

dodecyl sulphate (SDS) resulted in a marked decrease of surface LTA already at low concentrations whereas the β_2m binding was unaffected. Taken together these results show that surface LTA is not involved in the interaction of β_2m with group A streptococci.

Purified M12 protein was also used to study the interaction between M protein and β_2m . The M12 protein could partially inhibit the binding of aggregated HLA-antigens and β_2m to group A streptococci. In gel filtration of a mixture of β_2m and purified radio-labelled M12 protein about 20 per cent of the radioactivity was found in the V_0 peak corresponding to aggregated β_2m . Affinity chromatography of radiolabelled aggregated β_2m on Sepharose 4B coupled with M12 protein showed low but significant binding to the column.

The results related above suggest that M protein represents the surface structure of group A streptococci responsible for β_2m -binding. In our assays aggregates of β_2m and HLA-antigens are required indicating that a multipoint attachment is necessary for the interaction. It is therefore of interest that β_2m on the cell surface has been reported to be polymerized by tissue transglutaminase (33) which could mean that aggregates of β_2m are normally present on the cell surface in vivo. In our previous work it has been shown that LTA is the major cell surface component responsible for the hydrophobic affinity of group A streptococci. It has been suggested that LTA mediates the adhesion of streptococci to epithelial cells and to phagocytes (8, 12, 114, 116). We propose a hypothetical model where LTA through hydrophobic interactions enables the bacteria to come in close contact with the host cells and thereby facilitating a specific protein-protein interaction between M protein and β_2 -microglobulin.

General Summary

Recently, an increasing number of specific interactions between human serum proteins and gram-positive cocci have been reported. The biological significance of these bacteria-protein interactions is not clear. We have investigated the effect of specific binding of human serum proteins to bacteria on their surface properties as revealed by partition experiments in polymer two-phase systems. Pretreatment of bacteria with human serum resulted in an increase in surface hydrophobicity and less acidic isoelectric points of the organisms. Studies on the effect of individual proteins (human serum albumin, IgG, and fibrinogen) on the partition of the bacteria in polymer two-phase systems showed that a specific binding of protein to surface structures on gram-positive cocci changes the physicochemical surface properties of the bacteria.

Staphylococcal strains and streptococcal strains belonging to groups A, C, and G were investigated with respect to surface charge and hydrophobicity. Isoelectric points of the bacteria were determined by cross-partition in polymer two-phase systems. The results obtained indicated that group A, C, and G streptococci have isoelectric points around pH 3.75 and that there are no significant differences between individual strains. Staphylococci showed an isoelectric point of around pH 2 and thereby differed markedly from the streptococci.

The relative surface hydrophobicity of gram-positive cocci was also investigated in polymer two-phase systems. Streptococci

showed a large variation in hydrophobic affinity whereas all staphylococci investigated showed a low hydrophobic interaction liability. The hydrophobic structure on the surface of group A streptococci was characterized through the differential effect of the action of proteolytic enzymes on the surface hydrophobicity of the bacteria. The results obtained indicated that lipoteichoic acid (LTA) is the major cell wall component responsible for surface hydrophobicity of group A streptococci. This was further supported by the similarity of partition in polymer two-phase systems between whole group A streptococci and radiolabelled solubilized LTA. The content of surface LTA was also determined on whole group A streptococci by a novel method measuring the adsorption of antibodies to LTA to the bacterial surface. A correlation was found between the surface hydrophobicity of group A streptococci as measured in polymer two-phase systems and the content of surface LTA.

The possible contribution of M protein to the surface hydrophobicity of group A streptococci was also investigated. Surface LTA content and surface hydrophobicity was determined on a collection of M protein positive and M protein negative strains. Bacteria with a high hydrophobic affinity and a high content of surface LTA were found among both M-positive and M-negative strains and, in addition, several M-positive strains showed a low hydrophobic interaction liability. These results indicated that the antiphagocytic moiety of M protein does not contribute to the hydrophobic affinity of the bacteria. The development of a new assay for type-specific M antigens on whole group A streptococci enabled a study of the antigenic part of the M-molecule in situ. The results obtained demonstrated that the antigenic moiety of M protein is irrelevant to the surface hydrophobicity of group A streptococci.

Studies on the interaction between aggregated β_2 -microglobulin

(β_2m) and group A streptococci confirmed that M protein represents the bacterial surface structure responsible for β_2m -binding. β_2m is expressed at the cell surface of all nucleated cells and represents the invariant light chain of the major histocompatibility complex class I antigens. This topological localization of β_2m on human cells suggests that it might represent a specific binding molecule for group A streptococci in vivo. A hypothetical model is proposed where surface LTA enables the bacteria through hydrophobic interactions to overcome the electrical repulsion of the host cell thereby facilitating a specific interaction between M protein and β_2 -microglobulin.

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References

1. Albertsson, P.-Å. 1970. Partition of cell particles and macromolecules in polymer two-phase systems. *Adv. Prot. Chem.* 24: 309-341.
2. Albertsson, P.-Å. 1971. Partition of cell particles and macromolecules. Wiley-Interscience, New York.
3. Albertsson, P.-Å., S. Sasakawa, and H. Walter. 1970. Cross partition and isoelectric points of proteins. *Nature* 228: 1329-1330.
4. Alkan, M., and E. Beachey. 1978. Excretion of lipoteichoic acid by group A streptococci. *J. Clin. Invest.* 61: 671-676.
5. Alkan, M.L., J. Ofek, and E.H. Beachey. 1977. Adherence of pharyngeal and skin strains of group A streptococci to human skin and oral epithelial cells. *Infect. Immun.* 18: 555-557.
6. Armstrong, J.J., J. Baddiley, J.G. Buchanan, B. Carrs, and G.R. Greenberg. 1958. Isolation and structure of ribitol phosphate derivatives (teichoic acids) from bacterial cell walls. *J. Chem. Soc. p.* 4344-4354.
7. Armstrong, J.J., J. Baddiley, J.G. Buchanan, A.L. Davison, M.V. Kelemen, and F.C. Neuhaus. 1959. Teichoic acids from bacterial walls. Composition of teichoic acids from a number of bacterial walls. *Nature (London)* 184: 247-248.
8. Beachey, E.H. 1975. Binding of group A streptococci to human oral mucosal cells by lipoteichoic acid. *Trans. Assoc. Am. Physicians.* 88: 285-292.
9. Beachey, E.H. 1981. Bacterial adherence: Adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *J. Infect. Dis.* 143: 325-345.
10. Beachey, E.H., T.M. Chiang, I. Ofek, and A.H. Kang. 1977. Interaction of lipoteichoic acid of group A streptococci with human platelets. *Infect. Immun.* 16: 649-654.
11. Beachey, E.H., J.B. Dale, W.A. Simpson, J.B. Evans, K.W.

- Knox, I. Ofek, and A.J. Wicken. 1979. Erythrocyte binding properties of streptococcal lipoteichoic acids. *Infect. Immun.* 23: 618-625.
12. Beachey, E.H., and I. Ofek. 1976. Epithelial cell binding of group A streptococci by lipoteichoic acid on fimbriae denuded of M protein. *J. Exp. Med.* 143: 759-771.
 13. Beachey, E.H., and W. A. Simpson. 1982. The adherence of group A streptococci to oropharyngeal cells: the lipoteichoic acid adhesin and fibronectin receptor. *Infection* 10: 107-111.
 14. Beachey, E.H., and G.H. Stollerman. 1971. Toxic effects of streptococcal M protein on platelets and polymorphonuclear leucocytes in human blood. *J. Exp. Med.* 134: 351-365.
 15. Beachey, E.H., G.H. Stollerman, R.H. Johnson, I. Ofek, and A.L. Bisno. 1979. Human immune response to immunization with a structurally defined polypeptide fragment of streptococcal M protein. *J. Exp. Med.* 150: 862-877.
 16. Berggård, I., and A.G. Bearn. 1968. Isolation and properties of a low molecular weight β_2 -globulin occurring in human biological fluids. *J. Biol. Chem.* 243: 4095-4103.
 17. Björck, L., S.K. Tylewska, T. Wadström, and G. Kronvall. 1981. β_2 -microglobulin is bound to streptococcal M protein. *Scand. J. Immunol.* 13: 391-394.
 18. Björck, L., S.K. Tylewska, T. Wadström, and G. Kronvall. 1982. β_2 -microglobulin and its relation to streptococcal M protein and adherence, p 220-222. In S.E. Holm and P. Christensen (eds.), *Basic concepts of streptococci and streptococcal diseases*. Reedbooks Ltd., Chertsey.
 19. Bloom, B. 1979. Games parasites play: How parasites evade immune surveillance. *Nature* 279: 21-26.
 20. Carruthers, M.M., and W.J. Kabat. 1983. Mediation of staphylococcal adherence to mucosal cells by lipoteichoic acid. *Infect. Immun.* 40: 444-446.
 21. Christensen, P., and G. Kronvall. 1974. Capacity of group A, B, C, D, and G streptococci to agglutinate sensitized sheep red cells. *Acta Path. Microbiol. Scand. Sect. B.* 82: 19-24.
 22. Christensen, P., and V.-A. Oxelius. 1975. A reaction between some streptococci and IgA myeloma proteins. *Acta Path. Micro-*

23. Clegg, J.A., S.R. Smithers, and R.J. Terry. 1971. Acquisition of human antigens by Schistosoma mansoni during cultivation in vitro. Nature 232: 653-655.
24. Colleen, S., B. Hovelius, Å. Wieslander, and P.-Å. Mårdh. 1979. Surface properties of Staphylococcus saprophyticus and Staphylococcus epidermidis as studied by adherence test and two-polymer, aqueous phase system. Acta Path. Microbiol. Scand. Sect. B, 87: 321-328.
25. Corpe, W.A. 1970. Attachment of marine bacteria to solid surfaces, pp73-87. In R.S. Manly (ed.), Adhesion in biological systems. Academic Press, New York, London.
26. Cunningham, M.W., and E.H. Beachey. 1974. Peptic digestion of streptococcal M protein I. Effect of digestion at suboptimal pH upon the biological and immunochemical properties of purified M protein extracts. Infect. Immun. 9: 244-260.
27. Edebo, L., E. Kihlström, K.-E. Magnusson, and O. Stendahl. 1980. The hydrophobic effect and charge effects in the adhesion of enterobacteria to animal cell surfaces, and the influences of antibodies of different immunoglobulin classes, p. 65-101. In A.S.G. Curtis and H. Pitts (eds.), Cell adhesion and motility. Cambridge University Press, Cambridge.
28. Ellen, R.P., and R.J. Gibbons. 1972. M-protein-associated adherence of Streptococcus pyogenes to epithelial surfaces: prerequisite for virulence. Infect. Immun. 5: 826-830.
29. Endresen, C. 1978. Protein A reactivity of whole rabbit IgG and of fragments of rabbit IgG. Acta Path. Microbiol. Scand. Sect. C, 86: 211-214.
30. Ericson, I. 1974. Determination of the isoelectric point of rat liver mitochondria by cross-partition. Biochim. Biophys. Acta. 356: 100-107.
31. Eriksson, E., P.-Å. Albertsson, and G. Johansson. 1976. Hydrophobic surface properties of erythrocytes studied by affinity partition in aqueous two-phase systems. Mol. Cell. Biochem. 10: 123-128.
32. Erntell, M., E.B. Myhre, and G. Kronvall. 1983. Alternative non-immune F(ab')₂-mediated immunoglobulin binding to group C and G streptococci. Scand. J. Immunol. 17: 201-209.

33. Fesus, L., A. Falus, A. Erdei, and K. Laki. 1981. Human β_2 -microglobulin is a substrate of tissue transglutaminase: Polymerization in solution and on the cell surface. *J. Cell. Biol.* 89: 706-710.
34. Fiedel, B.A., and R.W. Jackson. 1978. Activation of the alternate complement pathway by a streptococcal lipoteichoic acid. *Infect. Immun.* 22: 286-287.
35. Fischetti, V.A. 1977: Streptococcal M protein extracted by nonionic detergent. II. Analysis of the antibody response to the multiple antigenic determinants of the M-protein molecule. *J. Exp. Med.* 146: 1108-1123.
36. Fischetti, V.A., E.C. Gotschlich, G. Siviglia, and J.B. Zabriskie. 1976. Streptococcal M protein extracted by nonionic detergent. I Properties of the antiphagocytic and type-specific molecules. *J. Exp. Med.* 144: 32-53.
37. Fischetti, V.A., J.B. Zabriskie, and E.C. Gotschlich. 1974. Physical, chemical, and biological properties of type 6 M protein extracted with purified streptococcal phage-associated lysin, p 26-36. *In* M.J. Haverkorn (ed.), *Streptococcal Disease and the Community*, Excerpta Medica, Amsterdam.
38. Forsgren, A., and J. Sjöquist. 1966. Protein A from *S. aureus*. I. Pseudo-immune reaction with human γ -globulin. *J. Immun.* 97: 822-827.
39. Fox, E.N. 1974. M proteins of group A streptococci. *Bact. Rev.* 38: 57-86.
40. Fox, E.N., and M.K. Wittner. 1969. New observations on the structure and antigenicity of the M proteins of the group A streptococcus. *Immunochem.* 6: 11-24.
41. Gibbons, R.J. 1977. Adherence of bacteria to host tissue. p. 395-406. *In* D. Schlessinger (ed.), *Microbiology*. American Society for Microbiology, Washington, D.C.
42. Goding, J.W. 1978. Use of staphylococcal protein A as an immunological reagent. *J. Immunol. Methods.* 20: 241-253.
43. Grey, H.M., R.T. Kubo, S.M. Colon, M.D. Poulik, P. Cresswell, T. Springer, M. Turner, and J.L. Strominger. 1973. The small subunit of HL-A antigens is β_2 -microglobulin. *J. Exp. Med.* 138: 1608-1612.
44. Griffith, F. 1934. The serological classification of *Strept-*

Streptococcus pyogenes . J. Hyg. (Camb.). 34: 542-585.

45. Halbert, S.P., and S.L. Keatinge. 1961. The analysis of streptococcal infections. VI. Immuno-electrophoretic observations on extracellular antigens detectable with human antibodies. J. Exp. Med. 113: 1013-1028.
46. Harboe, M., and I. Fölling. 1974. Recognition of two distinct groups of human IgM and IgA based on different binding to staphylococci. Scand. J. Immunol. 3: 471-482.
47. Hasty, D.L., E.H. Beachey, W.A. Simpson, and J.B. Dale. 1982. Hybridoma antibodies against protective and nonprotective antigenic determinants of a structurally defined polypeptide fragment of streptococcal M protein. J. Exp. Med. 155: 1010-1018.
48. Hausmann, E.O., O Lüderitz, K. Knox, A. Wicken, and N. Weinfield. 1975. Structural requirements for bone resorption by endotoxin and lipoteichoic acid. J. Dent. Res. 54: B94-B99.
49. Heath, T.-D., B.A. Macher, and D. Papahadjopoulos. Covalent attachment of immunoglobulins to liposomes via glycosphingolipids. Biochim. Biophys. Acta. 640: 66-81.
50. Hewett, M.J., K.W. Knox, and A.J. Wicken. 1970. Studies on the group F antigen of lactobacilli: detection of antibodies by haemagglutination. J. Gen. Microbiol. 60: 315-322.
51. Hill, M.J., A.M. James, and W.R. Maxted. 1962. Some physical investigations of the behaviour of bacterial surfaces. VIII. Studies on the capsular material of Streptococcus pyogenes . Biochim. Biophys. Acta. 66: 264-274.
52. Hirsch, J.G., and A.B. Church. 1960. Studies of phagocytosis of group A streptococci by polymorphonuclear leukocytes in vitro. J. Exp. Med. 111: 309-322.
53. Hjérten, S. 1976. Hydrophobic interaction chromatography of proteins on neutral adsorbents, p.233-243. In N. Catsimopoulos (ed), Methods of cell separation. Plenum Publishing Corp., New York.
54. Horie, S., H. Ishii, H. Nakazawa, T. Suga, and H. Oori. 1979. Determination of the cross-points of rat liver peroxisomes, peroxisomal core and the core components by cross-partition. Biochim. Biophys. Acta. 585: 435-443.
55. Hunter, W.H., and F.C. Greenwood. 1962. Preparation of

iodine-131 labelled human growth hormone of high specific activity. Nature (London) 194: 495-496.

56. Inganäs, M. 1981. Comparison of mechanisms of interaction between protein A from Staphylococcus aureus and human monoclonal IgG, IgA and IgM in relation to the classical Fc and the alternative Fab' ₂ protein A interactions. Scand. J. Immunol. 13: 343-352.
57. Jackson, D.E., C.R. Howlett, A.J. Wicken, and C.D.F. Jackson. 1981. Induction of hypersensitivity reactions to Lactobacillus fermentum and lipoteichoic acid in rabbits. Int. Archs. Allergy Appl. Immun. 65: 304-312.
58. Johansson, G. 1976. The effect of poly(ethylene glycol) esters on the partition of proteins and fragmented membranes in aqueous biphasic systems. Biochim. Biophys. Acta 451: 517-529.
59. Johansson, G., and H. Westrin. 1978. Specific extraction of intact chloroplasts using aqueous biphasic systems. Plant. Sci. Lett. 13: 201-212.
60. Johnson, R.H., and K.L. Vosti. 1977. Purification and characterization of group A streptococcal T-1 antigen. Infect. Immun. 16: 867-875.
61. Jonsson, P., and T. Wadström. 1983. High surface hydrophobicity of Staphylococcus aureus as revealed by hydrophobic interaction chromatography. Current Microbiol. 8: 347-353.
62. Joseph, R., and G.D. Shockman. 1975. The synthesis and excretion of glycerol teichoic acid during growth of two streptococcal species. Infect. Immun. 12: 333-338.
63. Kantor, F.S. 1965. Fibrinogen precipitation by streptococcal M protein. I. Identity of the reactants, and stoichiometry of the reaction. J. Exp. Med. 121: 849-859.
64. Käppeli, O., and A. Fiechter. 1976. The mode of interaction between the substrate and cell surface of the hydrocarbon-utilizing yeast Candida tropicalis. Biotechnol. Bioeng. 18: 974-976.
65. Kass, E.H., and C.V. Seastone. 1944. The role of the mucoid polysaccharide (hyaluronic acid) in the virulence of group A hemolytic streptococci. J. Exp. Med. 79: 310-330.
66. Kemp, W.M., S.C. Meritt, M.S. Boguchi, J.C. Rosier, and J.R.

- Seed. 1977. Evidence for absorption of heterospecific host immunoglobulin on the tegmentum of Schistosoma mansoni. J. Immunol. 119: 1849-1854.
67. Kessler, R.E., and G.D. Shockman. 1979. Precursorproduct relationship of intracellular and extracellular lipoteichoic acids of Streptococcus faecium. J. Bacteriol. 137: 869-877.
68. Kihlström, E., and L. Edebo. 1976. Association of viable and inactivated Salmonella typhimurium 395MS and MR10 with HeLa cells. Infect. Immun. 14: 851-857.
69. Kjellberg, S., C. Lagercrantz, and T. Larsson. 1980. Quantitative analysis of bacterial hydrophobicity studied by the binding of dodecanoic acid. FEMS Microbiol. Lett. 7: 41-44.
70. Knox, K.W., and A.J. Wicken. 1973. Immunological properties of teichoic acids. Bacteriol. Rev. 37: 215-257.
71. Köhler, W., and O. Prokop. 1978. Relationship between haptoglobin and Streptococcus pyogenes T4 antigens. Nature (London) 271: 373.
72. Krause, R.M. 1963. Antigenic and biochemical composition of hemolytic streptococcal cell wall. Bacteriol. Rev. 27: 369-380.
73. Krause, R.M., and C.H. Rammelkamp. 1962. Studies of the carrier state following infection with group A streptococci. II. Infectivity of streptococci isolated during acute pharyngitis and during the carrier state. J. Clin. Invest. 41: 575-578.
74. Kronvall, G. 1973. A surface component in group A, C and G streptococci with non-immune reactivity for immunoglobulin G. J. Immunol. 111: 1401-1406.
75. Kronvall, G., L. Björck, H. Miörner, E. Myhre, and K. Widebäck. 1982. Receptors for mammalian proteins in streptococcal species of human and animal origin, p. 219-220. In S.E. Holm and P. Christensen (eds.), Basic concepts of streptococci and streptococcal diseases. Reedbooks Ltd., Chertsey.
76. Kronvall, G., E.B. Myhre, L. Björck, and I. Berggård. 1978. Binding of aggregated human β_2 -microglobulin to surface protein structure in group A, C, and G streptococci. Infect. Immun. 22: 136-142.

77. Kronvall, G., C. Schönbeck, and E.B. Myhre. 1979. Fibrinogen binding structures in β -hemolytic streptococci group A, C and G. *Acta Path. Microbiol. Scand. Sect. B.* 87: 303-310.
78. Kronvall, G., A. Simmons, E. Myhre, and S. Jonsson. 1979. Specific absorption of human serum albumin, immunoglobulin A, and immunoglobulin G with selected strains of group A, and G streptococci. *Infect. Immun.* 25: 1-10.
79. Kühnemund, O., J. Havlicek, H. Knöll, and J. Sjöquist. 1981. M antigens of group A streptococci isolated by means of immunochromatography. Biochemical and serological properties. *Immunobiol.* 159: 244-255.
80. Kuusela, P. 1978. Fibronectin binds to Staphylococcus aureus. *Nature (London)* 276: 718-720.
81. Lancefield, R.C. 1928. The antigenic complex of Streptococcus hemolyticus. I. Demonstration of a type-specific substance in extracts of Streptococcus hemolyticus. *J. Exp. Med.* 47: 91-103.
82. Lancefield, R.C. 1933. A serological differentiation of human and other groups of hemolytic streptococci. *J. Exp. Med.* 57: 571-595.
83. Lancefield, R.C. 1954. Cellular constituents of group A streptococci concerned in antigenicity and virulence, p 3-18. In M McCarty (ed.), *Streptococcal Infections*, New York, Columbia Univ. Press.
84. Lancefield, R.C. 1957. Differentiation of group A streptococci with a common R antigen into three serological types, with special reference to the bactericidal test. *J. Exp. Med.* 106: 525-544.
85. Lancefield, R.C. 1958. Occurrence of R-antigen specific for group A, type 3 streptococci. *J. Exp. Med.* 108: 329-341.
86. Lancefield, R.C. 1959. Persistence of type-specific antibodies in man following infection with group A streptococci. *J. Exp. Med.* 110: 271-292.
87. Lancefield, R.C. 1962. Current knowledge of type-specific M antigens of group A streptococci. *J. Immunol.* 89: 307-313.
88. Lancefield, R.C., and G.E. Perlmann. 1952. Preparation and properties of a protein (R antigen) occurring in streptococci of group A, type 28 and in certain streptococci of other

serological groups. J. Exp. Med. 96: 83-97.

89. Laurell, C.-B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal. Biochem. 15: 45-52.
90. Leon, O., and C. Panos. 1983. Cytotoxicity and inhibition of normal collagen synthesis in mouse fibroblasts by lipoteichoic acid from Streptococcus pyogenes type 12. Infect. Immun. 40: 785-794.
91. Lindahl, M., A. Faris, T. Wadström, and S. Hjerten. 1981. A new test based on salting out to measure relative surface hydrophobicity of bacterial cells. Biochim. et Biophys. Acta. 677: 471-476.
92. Magnusson, K.-E. 1980. The hydrophobic effect and how it can be measured with relevance for cell-cell interactions. Scand. J. Infect. Dis. Suppl. 24: 131-134.
93. Magnusson, K.-E., J. Davies, T. Grundström, E. Kihlström, and S. Normark. Surface charge and hydrophobicity of Salmonella, E. coli and gonococci in relation to their tendency to associate with animal cells. Scand. J. Infect. Dis. Suppl. 24: 135-140.
94. Magnusson, K.-E., E. Kihlström, L. Norlander, A. Norqvist, J. Davies, and S. Normark. 1979. Effect of colony type and pH on surface charge and hydrophobicity of Neisseria gonorrhoeae. Infect. Immun. 26: 379-401.
95. Magnusson, K.-E., O. Stendahl, C. Tagesson, L. Edebo, and G. Johansson. The tendency of smooth and rough Salmonella typhimurium bacteria and lipopolysaccharide to hydrophobic and ionic interaction as studied in aqueous polymer two-phase systems. Acta Path. Microbiol. Scand. Sect. B, 85: 212-218.
96. Malmqvist, T. 1983. Bacterial hydrophobicity measured as partition of palmitic acid between the two immiscible phases of cell surface and buffer. Acta Path. Microbiol. Scand. Sect. B 91: 69-73.
97. Markham, J.L., K.W. Knox, A.J. Wicken, and M.J. Hewett. 1975. Formation of extracellular lipoteichoic acid by oral streptococci and lactobacilli. Infect. Immun. 12: 378-386.
98. Marshall, K.C., R. Stout, and R. Mitchell. 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. J. Gen. Microbiol. 68: 337-348.

99. Maxted, W.R. 1956. The indirect bactericidal test as a means of identifying antibody to the M antigen of Streptococcus pyogenes. Brit. J. Exp. Pathol. 37: 415-422.
100. McCarty, M. 1964. The role of D-alanine in the serological specificity of group A streptococcal glycerol teichoic acid. Microbiol. 52: 259-265.
101. McConahey, P.J., and F.J. Dixon. 1966. A method of trace iodination of protein for immunologic studies. Int. Arch. Allergy Appl. Immunol. 29: 185-189.
102. Michael, J.G., and B.F. Massell. 1965. Use of unabsorbed antisera in gel diffusion for grouping and typing of hemolytic streptococci. J. Lab. Clin. Med. 65: 322-328.
103. Miller, B.A., and R.W. Jackson. 1973. The effect of Streptococcus pyogenes teichoic acid on the immune response of mice. J. Immunol. 110: 148-156.
104. Milton, R.C. 1964. An extended table of critical values for the Mann-Whitney (Wilcoxon) two-sample statistic. J. Amer. Statist. Ass. 59: 925-934.
105. Myhre, E.B., O. Holmberg, and G. Kronvall. 1979. Immunoglobulin-binding structures on bovine group G streptococci different from type III Fc receptors on human group G streptococci. Infect. Immun. 23: 1-7.
106. Myhre, E.B., and G. Kronvall. 1977. Heterogeneity of non-immune immunoglobulin Fc reactivity among Gram-positive cocci: Description of three major types of receptors for human immunoglobulin G. Infect. Immun. 17: 475-482.
107. Myhre, E.B., and G. Kronvall. 1980. Demonstration of specific binding sites for human serum albumin in group C and G streptococci. Infect. Immun. 27: 6-14.
108. Myhre, E.B., and G. Kronvall. 1980. Demonstration of a new type of immunoglobulin G receptor in Streptococcus zooepidemicus strains. Infect. Immun. 27: 808-816.
109. Myhre, E.B., and P. Kuusela. 1983. Binding of human fibronectin to group A, C and G streptococci. Infect. Immun. 40: 29-34.
110. Nagura, H., J. Asai, Y. Katsumata, and K. Kojima. 1973. Role of electric surface charge of cell membrane in phagocytosis.

111. Nakamuro, K., N. Tanigaki, D. Pressman. 1973. Multiple common properties of human β_2 -microglobulin and the common portion fragment derived from HLA-A antigen molecules. Proc. Nat. Acad. Sci. (USA) 70: 2863-2865.
112. Nealon, T.J., and S.J. Mattingly. 1983. Association of elevated levels of cellular lipoteichoic acids of group B streptococci with human neonatal disease. Infect. Immun. 39: 1243-1251.
113. Nilsson, K., P.-E. Evrin, and K.I. Welsh. 1974. Production of β_2 -microglobulin by normal and malignant human cell lines and peripheral lymphocytes. Transplant. Rev. 21: 53-84.
114. Ofek, I., and E.H. Beachey. 1979. Lipoteichoic-acid-sensitive attachment of group A streptococci to phagocytes, p. 44-46. In M.T. Parker (ed.), Pathogenic streptococci. Reed-books, Ltd., Surrey, England.
115. Ofek, I., and E.H. Beachey. 1980. General concepts and principles of bacterial adherence in animals and man, p. 3-29. In E.H. Beachey (ed.), Bacterial adherence (Receptors and recognition) Ser. B. Vol. 6. Chapman & Hall, London.
116. Ofek, I., E.H. Beachey, W. Jeffersson, and G.L. Campbell. 1975. Cell membrane-binding properties of group A streptococcal lipoteichoic acid. J. Exp. Med. 141: 990-1003.
117. Ofek, I., W.A. Simpson, and E.H. Beachey. 1982. Formation of molecular complexes between a structurally defined M protein and acylated or deacylated lipoteichoic acid of Streptococcus pyogenes. J. Bacteriol. 149: 426-433.
118. Ofek, I., E. Whitnack, and E.H. Beachey. 1983. Hydrophobic interactions of group A streptococci with hexadecane droplets. J. Bacteriol. 154: 139-145.
119. Perers, L., L. Andåker, L. Edebo, O. Stendahl, and C. Tage-sson. 1977. Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. Acta Path. Microbiol. Scand. Sect. B, 85: 308-316.
120. Peterson, P.A., B.A. Cunningham, I. Berggård, and G.M. Edelman. 1972. β_2 -microglobulin - a free immunoglobulin domain. Proc. Natl. Acad. Sci. (USA) 69: 1697-1701.

121. Peterson, P.A., L. Rask, and J.B. Lindblom. 1974. Highly purified papain-solubilized HL-A antigens contain β_2 -microglobulin. *Proc. Nat. Acad. Sci. (USA)* 71: 35-39.
122. Phillips, G.N.Jr., P.F. Flicker, C.Cohen, B.N. Manjula, and V. A. Fischetti. 1981. Streptococcal M protein: α -Helical coiled-coil structure and arrangement on the cell surface. *Proc. Natl. Acad. Sci. (USA)* 78: 4689-4693.
123. Rolla, G., R.V. Oppermann, W.H. Bowen, J.E. Ciardi, and K.W. Knox. 1980. High amounts of lipoteichoic acid in sucrose-induced plaque in vivo. *Caries. Res.* 14: 235-238.
124. Rolla, G., S.A. Robrish, and W.H. Bowen. 1977. Interaction of hydroxyapatite and protein-coated hydroxyapatite with *Streptococcus mutans* and *Streptococcus sanguis*. *Acta Path. Microbiol. Scand. Sect. B*, 85: 311-346.
125. Rosa, U., G.A. Scassellati, F. Pennisi, N. Riccioni, P. Giagnoni, and R. Giordani. 1964. Labelling of human fibrinogen with ^{125}I by electrolytic iodination. *Biochem. Biophys. Acta* 86: 519-526.
126. Rosenberg, M., D. Gutnick, and E. Rosenberg. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.* 9: 29-33.
127. Rotta, J., R.M. Krause, R.C. Lancefield, W. Everly, and H. Lackland. 1971. New approaches for the laboratory recognition of M types of group A streptococci. *J. Exp. Med.* 134: 1298-1315.
128. Schalén, C. 1980. The group A streptococci receptor for human IgA binds IgA via the Fc-fragment. *Acta Path. Microbiol. Scand. Sect. C*, 88: 271-274.
129. Schleifer, K.H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* 36: 407-477.
130. Schönbeck, C., L. Björck, and G. Kronvall. 1981. Receptors for fibrinogen and aggregated β_2 -microglobulin detected in strains of group B streptococci. *Infect. Immun.* 31: 856-861.
131. Sher, A., B.F. Hall, and M.A. Vadas. 1978. Acquisition of murine histocompatibility complex gene products by schistosome of *Schistosoma mansoni*. *J. Exp. Med.* 148: 46-57.
132. Simpson, W.A., D.L. Hasty, J.M. Mason, and E.H. Beachey.

1982. Fibronectin-mediated binding of group A streptococci to human polymorphonuclear leukocytes. *Infect. Immun.* 37: 805-810.
133. Smith, H. 1977. Microbial surfaces in relation to pathogenicity. *Bacteriol. Rev.* 41: 475-500.
 134. Smithies, O., and M.D. Poulik. 1972. Initiation of protein synthesis at an unusual position in an immunoglobulin gene. *Science* 175: 187-189.
 135. Smyth, C.J., P. Jonsson, E. Olsson, O. Söderlind, J. Rosengren, S. Hjertén, and T. Wadström. 1978. Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. *Infect. Immun.* 22: 462-472.
 136. Stendahl, O., L. Edebo, K.-E. Magnusson, C. Tagesson, and S. Hjertén. 1977. Surface-charge characteristics of smooth and rough *Salmonella typhimurium* bacteria determined by aqueous two-phase partitioning and free zone electrophoresis. *Acta Path. Microbiol. Scand. Sect. B*, 85: 334-340.
 137. Stendahl, O., B. Normann, and L. Edebo. 1979. Influence of O and K antigens on the surface properties of *Escherichia coli* in relation to phagocytosis. *Acta Path. Microbiol. Scand. Sect. B*, 87: 85-91.
 138. Stendahl, O., C. Tagesson, and M. Edebo. 1973. Partition of *Salmonella typhimurium* in a two-polymer aqueous phase system in relation to liability to phagocytosis. *Infect. Immun.* 8: 36-41.
 139. Stollerman, G.H., and R. Ekstedt. 1957. Long chain formation by strains of group A streptococci in the presence of homologous antiserum: a type-specific reaction. *J. Exp. Med.* 106: 345-355.
 140. Stollerman, G.H., M. Rytel, and J. Ortiz. 1963. Accessory plasma factors involved in the bactericidal test for type-specific antibody to group A streptococci. *J. Exp. Med.* 117: 1-17.
 141. Sundler, R., and J. Wijkander. 1983. Protein-mediated inter-membrane contact specifically enhances Ca^{2+} -induced fusion of phosphatidate-containing membranes. *Biochim. Biophys. Acta* 730: 391-394.

142. Swanson, J., K.C. Hsu, and E.C. Gotschlich. 1969. Electron microscopic studies on streptococci. I. M antigen. J. Exp. Med. 130: 1063-1091.
143. Swanson, J., J.E. Sparks, D. Young, and G. King. 1975. Studies on gonococcus infection. X. Pili and leukocytes association factor as mediators of interactions between gonococci and eukaryotic cells in vitro. Infect. Immun. 11: 1352-1361.
144. Swift, H.F., A.T. Wilson, and R.C. Lancefield. 1943. Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. J. Exp. Med. 78: 127-133.
145. Switalski, L.M., Å. Ljungh, C. Rydén, K. Rubin, M. Höök, and T. Wadström. 1982. Binding of fibronectin to the surface of group A, C, and G streptococci isolated from human infections. Eur. J. Clin. Microbiol. 1: 381-387.
146. Szoka, F., and D. Papahadjopoulos. 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. (USA) 75: 4194-4198.
147. Tanford, C. 1980. The hydrophobic effect. Formation of micelles and biological membranes, 2nd ed., Wiley & Sons, New York.
148. Tillett, W.S., and R.L. Garner. 1934. The agglutination of hemolytic streptococci by plasma and fibrinogen. A comparison of the phenomenon to serological reactions with the same organisms. Bull. John Hopkins Hosp. 54: 145-156.
149. Todd, E.W., and R.C. Lancefield. 1928. Variants of hemolytic streptococci; their relation to type-specific substance, virulence, and toxin. J. Exp. Med. 48: 751-767.
150. Torpier, G., A. Capron, and M.A. Quaiissi. 1979. Receptor for IgG (Fc) and human β_2 -microglobulin on S. mansoni schistosomula. Nature 278: 447-449.
151. Tylewska, S., S. Hjertén, and T. Wadström. 1979. Contribution of M protein to the hydrophobic surface properties of Streptococcus pyogenes. FEMS Microbiol. Lett. 6: 249-253.
152. Tylewska, S.K., S. Hjertén, and T. Wadström. 1980. Hydrophobic properties of Streptococcus pyogenes related to M protein: Decrease of surface hydrophobicity and epithelial cell binding by antibiotics, p. 788. In J.D. Nelson and C.

Grassi (eds.), Current chemotherapy and infectious diseases, vol. 2. American Society for Microbiology, Washington, D.C.

153. Van Driel, D., A.J. Wicken, M.R. Dickson, and K.W. Knox. 1973. Cellular location of the lipoteichoic acids of Lactobacillus fermenti NCTC 6991 and Lactobacillus casei NCTC 6375. J. Ultrastruct. Res. 43: 483-497.
154. Van Oss, C.J. 1978. Phagocytosis as a surface phenomenon. Ann. Rev. Microbiol. 32: 19-39.
155. Van Oss, C.J., C.F. Gillman, and A.W. Neumann. 1975. Phagocytic engulfment and cell adhesiveness as cellular surface phenomena. Marcel-Dekker, New York.
156. Vosti, K., R.H. Johnson, and M.F. Dillon. 1971. Further characterization of purified fractions of M protein from a strain of group A type 12 streptococcus. J. Immunol. 107: 104-114.
157. Wadström, T., C.J. Smyth, A. Faris, P. Jonsson, and J. Freer. 1978. Hydrophobic adsorptive and hemagglutinating properties of enterotoxigenic Escherichia coli with different colonization factor antigens and adherence factor, p. 29-43. In Neonatal Diarrhoea, VIDO Symposium, Stuart Bundle Printing Press, Saskatoon, Canada.
158. Wadström, T., and S. Tylewska. 1982. Surface hydrophobicity of group A streptococci. J. Infect. Dis. 146: 576.
159. Wagner, M., B. Wagner, G. Kronvall, and L. Björck. 1983. Electron microscopic localization of receptors for aggregated β_2 -microglobulin on the surface of beta-hemolytic streptococci. Infect. Immun. In press.
160. Walter, H., E.J. Krob, and R. Tung. 1976. Hydrophobic affinity partition in aqueous two-phase systems of erythrocytes from different species. Exp. Cell. Res. 102: 14-24.
161. Ward, J.B. 1981. Teichoic and teichuronic acids: Biosynthesis, assembly, and location. Microbiol. Rev. 45: 211-243.
162. Westphal, O., O. Luderitz, and F. Bister. 1952. Über die extraktion von bakterien mit phenol/wasser. Z. Naturforsch. 7b: 148-155.
163. Westrin, H., P.-Å. Albertsson, and G. Johansson. 1976. Hydrophobic affinity partition of spinach chloroplasts in aqueous two-phase systems. Biochim. Biophys. Acta 436: 696-706.

164. Whitnack, E., A.L. Bisno, and E.H. Beachey. 1981. Hyaluronate capsule prevents attachment of group A streptococci to mouse peritoneal macrophages. *Infect. Immun.* 31: 985-991.
165. Wicken, A.J., K.W. Broady, A. Ayres, and K.W. Knox. 1982. Production of lipoteichoic acid by lactobacilli and streptococci grown in different environments. *Infect. Immun.* 36: 864-869.
166. Wicken, A.J., J.W. Gibbens, and K.W. Knox. 1973. Comparative studies on the isolation of membrane lipoteichoic acid from Lactobacillus fermenti. *J. Bacteriol.* 113: 365-372.
167. Wicken, A.J., and K.W. Knox. 1970. Studies on the group F antigen of lactobacilli: Isolation of a teichoic acid-lipid complex from Lactobacillus fermenti NCTC 6991. *J. Gen. Microbiol.* 60: 293-301.
168. Wicken, A.J. and K.W. Knox. 1975. Lipoteichoic acids: A new class of bacterial antigen. *Science* 187: 1161-1167.
169. Wicken, A.J., and K.W. Knox. 1977. Biological properties of lipoteichoic acids, p. 360-365. *In* D. Schlessinger (ed.), *Microbiology*. American Society for Microbiology, Washington, D.C.
170. Widdowson, J.P., W.R. Maxted, and A.M. Pinney. 1971. An M-associated protein antigen (MAP) of group A streptococci. *J. Hyg.* 69: 553-564.
171. Widebäck, K., J. Havlicek, and G. Kronvall. Demonstration of a receptor for mouse and human serum albumin in Streptococcus pyogenes. *Acta Path. Microbiol. Scand. Sect. B.* In press.
172. Widebäck, K., and G. Kronvall. 1982. Surface receptors for serum albumin in group C and G streptococci show three different types of albumin specificity. *Infect. Immun.* 38: 1154-1163.
173. Wittner, M.K., and E.N. Fox. 1971. Micro complement fixation assay for type-specific group A streptococcal antibody. *Infect. Immun.* 4: 441-445.
174. ZoBell, C.E. 1943. The effect of solid surfaces upon bacterial activity. *J. Bacteriol.* 46: 39-59

APPENDIX: PAPERS I–VI

Effect of Specific Binding of Human Albumin, Fibrinogen, and Immunoglobulin G on Surface Characteristics of Bacterial Strains as Revealed by Partition Experiments in Polymer Phase Systems

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Four strains of gram-positive cocci with different combinations of positive binding of human proteins were investigated with respect to changes in physicochemical surface properties after specific protein binding. *Staphylococcus aureus* Cowan I, two group A β -hemolytic streptococci, and one group G streptococcal strain were studied; they represented three different combinations of reactivity for human serum albumin, human immunoglobulin G, and fibrinogen. Using single-tube partition of bacterial cells in a dextran-polyethylene glycol system of constant polymer concentration but varying ionic compositions, it was possible to detect changes in the partition of bacteria after specific protein binding. There was a correlation between the binding of radiolabeled human proteins to the bacterial strains and the effect of human proteins on the partition of the bacteria in the phase systems. Thus, the specific binding of proteins to the bacteria changes their physicochemical surface properties. These types of bacteria-protein interactions may play an important role in modulating host-parasite relationships.

The surface characteristics of bacteria play an important role in pathogenicity (32). Several factors seem to modulate cell surface charge and degree of hydrophobicity. Recently, an increasing number of specific interactions between surface structures of streptococci and human proteins have been discovered. Fibrinogen-binding properties were found first in some group A streptococci but are now known to reside in all group A, C, and G strains (9, 13, 19, 39). Immunoglobulin G (IgG) and IgA binding seems to depend on separate surface structures (7, 16, 17, 25). A binding of aggregated β_2 -microglobulin to group A, C, and G streptococci has been demonstrated (17, 18). Haptoglobin shows affinity for some streptococci (15). Albumin binds to a specific receptor on group C and G streptococci (20, 26). *Staphylococcus aureus* organisms are also known to bind various immunoglobulins as well as fibrinogen (11, 24). The high density of receptors for some of these proteins suggests that a positive protein binding might influence the physicochemical properties of the bacterial cell surface.

Partition between two immiscible liquid phases composed of different aqueous polymer solutions has been used to investigate surface properties of *Salmonella typhimurium* and *Escherichia coli* (21, 22, 34-37). Particles, such as bacteria, distribute between the two phases and

the interface in such aqueous phase systems (1-3). Factors determining the partition include several more or less independent variables such as surface charge, hydrophobic-hydrophilic composition of the particle, and spatial conformation (3).

To study the effects of protein-bacteria interactions on surface characteristics, we used single-tube partition of bacterial cells in a dextran-polyethylene glycol (PEG) system of constant polymer concentrations with varying ionic compositions. The results obtained indicate that the presence of receptors for the proteins studied influences the partition of the bacteria. These types of bacteria-protein interactions may play an important role in modulating the host-parasite relationship.

MATERIALS AND METHODS

Bacterial strains. Strains with different combinations of positive binding of human proteins were selected for partition studies and included one *S. aureus* strain (Cowan I), two group A streptococcal strains (AM1 and AW43), and one group G strain (G148). As shown in Table 1, these strains represented three different combinations of reactivity for the proteins studied. One strain, G148, showed specific binding of human serum albumin (20, 26). All strains showed specific binding of human IgG (16, 20, 25), whereas only the group A streptococci and *S. aureus* showed specific binding of fibrinogen (19). All strains

were kept at 4°C on blood agar plates before use. Tryptone broth or Todd-Hewitt broth was inoculated and incubated overnight at 37°C. Bacteria were harvested by centrifugation at 3,000 rpm for 15 min and washed twice in phosphate-buffered saline (0.03 M phosphate, 0.12 M NaCl, pH 7.2). The optical density at 540 nm was measured, and the bacterial concentration was calculated from a standard curve and adjusted to 10⁹ organisms per ml.

Radiolabeling of bacteria. Bacteria were labeled with ⁵¹Cr (The Radiochemical Centre, Amersham, England). Fifty microCuries of ⁵¹Cr was added to 10⁹ bacteria in phosphate-buffered saline containing 0.05% Tween 20 and incubated at 37°C for 4 h. The bacteria were then deposited by centrifugation, washed four times in phosphate-buffered saline-Tween, and suspended in sterile water to contain 2.5 × 10⁸ bacteria per ml. Radioactivity in the last supernatant never exceeded 2% of that in the pellet. Radioactivity per bacterial cell was in the range of 10⁻³ cpm.

Human protein preparations. Human serum albumin and human fibrinogen were purchased from Kabi AB (Stockholm, Sweden). IgG1 myeloma protein was obtained as previously described (16). Human, mouse, and fetal bovine sera used were pools of normal serum samples. Bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, Mo. Proteins and sera were diluted in sterile water.

Radiolabeling of human albumin, IgG, and fibrinogen. Human serum albumin and IgG were labeled with ¹²⁵I (The Radiochemical Centre) by using

the chloramine-T method (23). Fibrinogen was radio-labeled by using the electrolytic method of Rosa et al. (28) as described by Harboe and Fölling (12).

Polymer two-phase systems. PEG 6000 (Union Carbide, New York) and dextran T500 (Pharmacia Fine Chemicals, Uppsala, Sweden), separating into an upper and lower phase, respectively, were used for the liquid polymer phase system (2). The phase systems were prepared from a 20% (wt/wt) stock solution of dextran and a 40% (wt/wt) stock solution of PEG. Salt and buffer stock solutions were made in concentrations 10 times higher than that of the final concentration in the phase system. Single-tube partitions at constant polymer compositions were performed in plastic tubes (70 by 12 mm; AB CERBO, Trollhättan, Sweden). Total capacity of each system was 2 g. The polymer solutions were weighed in each tube, and appropriate buffer and salt solutions were then added with a carefully calibrated pipette (Oxford Laboratories, Foster City, Calif.). Partition of the bacteria more in favor of the lower phase was obtained through addition of NaCl (2).

To select a phase system suitable for each strain, a series of single-tube experiments were set up (Table 2). The phase composition selected for investigating the group G *Streptococcus* (G148) and the group A *Streptococcus* (AM1) was: 5% (wt/wt) dextran T500; 4% (wt/wt) PEG 6000; 0.01 M sodium phosphate; pH 7.8. For *S. aureus* and group A *Streptococcus* (AW43), the following composition was used: 5% (wt/wt) dextran T500; 4% (wt/wt) PEG 6000; 0.01 M lithium phosphate; pH 6.8.

Thus in a typical experiment 0.5 g of 20% dextran T500 and 0.2 g of 40% PEG 6000 were weighed in, and then 200 µl of 0.1 M buffer and 600 µl of sterile water were added. Adding 400 µl of bacteria and 100 µl of protein solution in sterile water to the phase system gave the desired final concentration of all components. All phase systems were prepared and experiments were carried out at room temperature.

Phase partition experiments. Initially, incubation of protein with bacteria was performed before adding the cells to the phase systems. The proteins were mixed with bacteria in phosphate-buffered saline, and the mixtures were allowed to stand at room temperature for 45 min. The bacteria were then washed twice, suspended in sterile water, and added to the phase system. Similar results were obtained when proteins and bacteria were added separately to the

TABLE 1. Binding of radiolabeled human proteins to bacterial strains

Bacterial strains	% Binding ^a		
	Human albumin	IgG	Fibrinogen
<i>S. aureus</i>	2	77	16
Group A <i>Streptococcus</i> , AW43	5	26	53
Group A <i>Streptococcus</i> , AM1	8	67	57
Group G <i>Streptococcus</i> , G148	70	75	7

^a Figures denote percent uptake of 0.4 µg of human serum albumin, 0.5 µg of human IgG, and 5.0 µg of fibrinogen to 2 × 10⁸ bacteria. The uptake is expressed as percentage of added radioactivity (16, 19, 20, 25, 26).

TABLE 2. Polymer two-phase systems with partition behavior of bacterial strains

Bacterial strains	Two-phase systems ^a											
	1			2			3			4		
	T	I	B	T	I	B	T	I	B	T	I	B
<i>S. aureus</i>	70.7	24.4	4.9	40.0	55.5	4.5	88.2	9.2	2.6	80.7	16.3	3.0
Group A <i>Streptococcus</i> ; AW43	3.7	55.4	40.9	2.7	35.6	61.7	5.8	88.8	4.4	29.9	66.2	3.9
Group A <i>Streptococcus</i> ; AM1	27.6	68.8	3.6	7.8	84.8	7.4	33.0	60.2	6.8	71.2	24.5	4.3
Group G <i>Streptococcus</i> ; G148	12.8	84.2	3.0	9.4	87.7	2.9	16.2	81.6	2.2	28.7	67.9	3.4

^a System 1, 5% (wt/wt) dextran, 4% (wt/wt) PEG 6000, 0.01 M sodium phosphate (pH 7.8); system 2, same except pH 6.8; system 3, 5% dextran, 4% PEG 6000, 0.01 M lithium phosphate (pH 6.8); system 4, 5% dextran, 3.6% PEG 6000, 0.01 M lithium phosphate (pH 6.8). Data indicate percentage of bacterial cells in PEG-rich top phase (T), interphase (I), and dextran-rich bottom phase (B). The bacteria were labeled with ⁵¹Cr. Boldface numbers indicate the phase into which the majority of the cells partition.

phase systems and the bindings were allowed to take place within the systems. Proteins, in amounts as indicated, were added in 100- μ l volumes, and 10^8 bacteria were added in 400- μ l volumes. The systems were mixed thoroughly by inverting the tubes about 50 times. After 45 min of settling time, sufficient for phase separation and maximal protein binding, 200 μ l of the top or bottom phase, or both, was withdrawn from each tube, and the radioactivity in each sample was measured in a gamma scintillation counter (LKB-Wallace 1280 Ultro Gamma; Biotec, Stockholm, Sweden). Percent bacterial cells in the top and the bottom phase was calculated. The distribution of bacteria is presented as percentage of cells in the dextran-rich bottom phase. In experiments determining the partition coefficient of proteins alone, 500 μ l of 125 I-labeled protein in sterile water was added to the phase systems. The partition coefficient (K) is defined as concentration in top phase divided by concentration in bottom phase.

RESULTS

Effect of serum on bacterial partition.

One hundred microliters of normal human serum, diluted 1:50 in sterile water, was added together with 10^8 bacterial organisms to the phase systems. The partition of all four strains studied was strongly influenced by the addition of serum (Fig. 1). The group G *Streptococcus* (G148) showed the largest change in partition behavior. At 40 mM NaCl, the addition of human serum resulted in a change in partition of the group G *Streptococcus* from 22 to 84% in the bottom phase (Fig. 1C). In a second series of experiments, normal human serum diluted from 1:50 to 1:640 was added to the group G *Streptococcus* and resulted in a dose-dependent transfer of the bacterial cells from the upper phase and the interphase into the bottom phase (Fig. 1D).

The effect of normal serum from other species was also studied. Fetal bovine serum had no influence on the partition of the two group A streptococci or the *S. aureus* strain. A slight change in the partition of the group G *Streptococcus* was obtained when tested with bovine serum (less than 10% as compared to more than 60% with the same amount of human serum). The addition of mouse serum to the group G *Streptococcus*, which is capable of binding murine IgG, resulted in a marked change in partition, in contrast to the lack of any influence on the partition of a group A streptococcal strain.

Effect of albumin on bacterial partition.

Human serum albumin (10 μ g per 10^8 bacteria) was added together with the bacterial cells to the phase systems. No effect was seen on the partition of the *S. aureus*, whereas a slight effect on the partition of the group A streptococci was seen at high NaCl concentrations (Fig. 2A and B). In contrast, human serum albumin had a strong effect on the partition of the group G streptococcus (Fig. 2C).

Experiments using varying concentrations of human serum albumin were also performed. As seen in Fig. 2D, low concentrations of albumin had an early marked effect on the partition of the group G *Streptococcus*. At 40 mM NaCl, the addition of 1.5 μ g of human serum albumin per 10^8 bacteria resulted in a change in partition from 38 to 67% in the bottom phase. The addition of different concentrations showed that the influence is a dose-dependent and saturable process.

Bovine albumin does not bind to the receptor for human albumin in strain G148. Control experiments were therefore performed to measure the effect of bovine serum albumin on the dis-

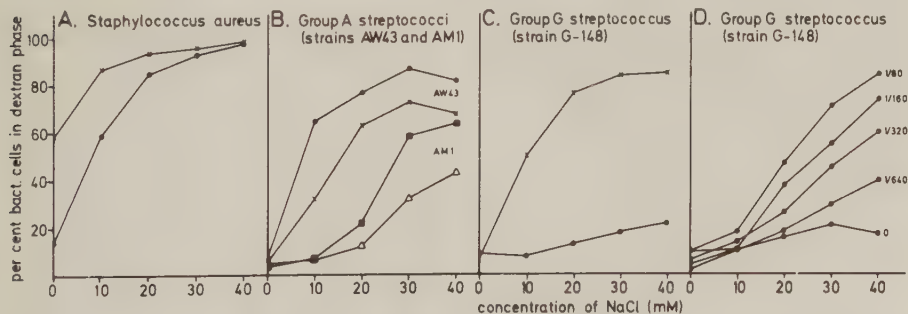


FIG. 1. Effect of normal human serum on partition of bacterial cells in the dextran-PEG phase system at constant polymer concentrations but different ionic compositions. Symbols: (●, ■) bacterial cells only; (×, △) bacterial cells with human serum added at a 1:50 dilution (A, B, and C). The effect of varying dilutions of serum on strain G148 is shown in (D). In Fig. 1A, 2A, 3A, 3B, and 4A the distribution is presented as percent bacterial cells in dextran phase plus interface, as calculated by the subtraction of percent in the PEG phase from the total 100%.

tribution of the bacterial cells in the phase systems. Bovine albumin had no effect on the partition of the group A streptococci or *S. aureus*, but it had a slight effect on the partition of the group G *Streptococcus*, equivalent to the effect of fetal bovine serum.

To study the partition of protein alone, ^{125}I -labeled human serum albumin was added to the systems. An increase in NaCl concentration resulted in a slight decrease of the partition coefficient in the systems used (a decrease in K value from 1.7 to 0.68).

Effect of IgG on bacterial partition. Ten micrograms of human IgG1 myeloma protein was added together with 10^8 bacterial cells to the phase systems. As shown in Fig. 3, the addition of IgG influenced the partition of all four strains studied. *S. aureus* Cowan I showed a marked change in partition (from 14 to 59%), whereas the other strains showed a lower but

definite change. The addition of increasing amounts of IgG (2.5 to 20 μg) to *S. aureus* revealed that the effect was dose dependent (Fig. 3B). The partition coefficient of radiolabeled soluble IgG was decreased through the addition of NaCl, a change opposite the effect of IgG when bound to the group A streptococci (a decrease in K value from 7.2 to 1.4).

Effect of fibrinogen on bacterial partition. The effect of 5 μg of human fibrinogen on the partition of 10^8 bacterial cells was studied. Fibrinogen had a low but definite influence on the distribution of *S. aureus* and the group A streptococci in the phase systems used (Fig. 4). There was no effect of fibrinogen on partition of the group G *Streptococcus* lacking a receptor for this protein (Table 1). A decrease in partition coefficient of radiolabeled fibrinogen was observed when the NaCl concentration was increased (a decrease in K value from 6.2 to 1.8).

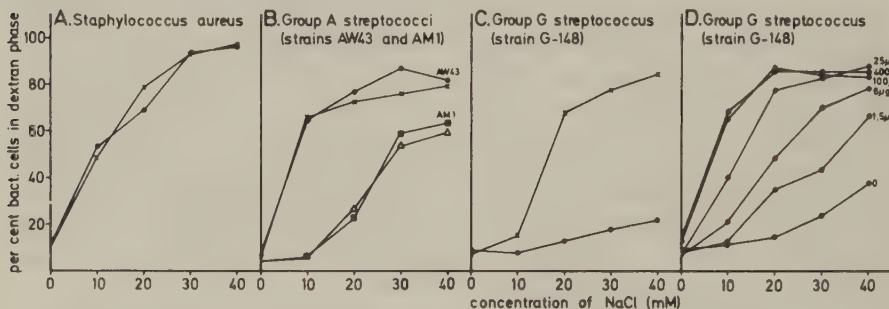


FIG. 2. Effect of human serum albumin on partition of bacterial cells in the dextran-PEG system at constant polymer concentrations but different ionic compositions. Symbols: (●, ■) bacterial cells only; (×, △) bacterial cells and 10 μg of human serum albumin added (A, B, and C). The effect of varying concentrations of human serum albumin on strain G148 is shown in (D).

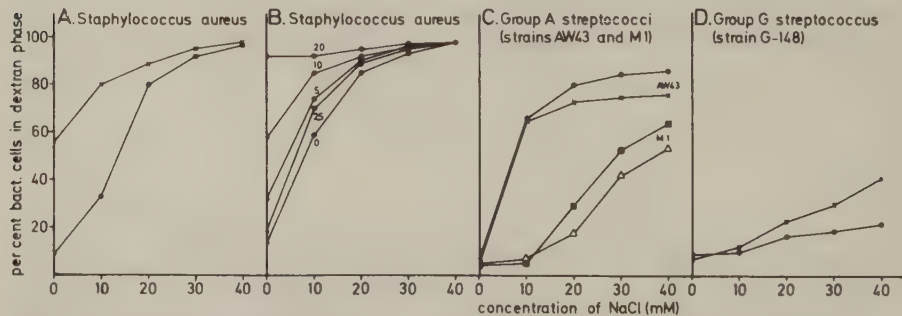


FIG. 3. Effect of human IgG1 myeloma protein on partition of bacterial cells in the dextran-PEG system at constant polymer concentrations but different ionic compositions. Symbols: (●, ■) bacterial cells only; (×, △) bacterial cells and 10 μg of IgG1 added (A, C, and D). (B) Effect of varying concentrations of human IgG1 on *S. aureus*.

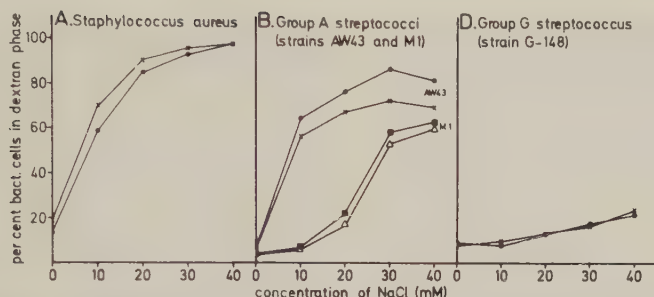


FIG. 4. Effect of human fibrinogen on partition of bacterial cells in the dextran-PEG system at constant polymer concentrations but different ionic compositions. Symbols: (●, ■) bacterial cells only; (×, △) bacterial cells and 5 μ g of fibrinogen added.

DISCUSSION

The present studies describe marked changes in surface characteristics of four bacterial strains after specific binding of human proteins as revealed by partition in polymer phase systems. The bacteria used carry surface receptors for human serum albumin, IgG, and fibrinogen in different combinations (9, 11, 13, 16, 19, 20, 24-26, 39). As seen in Tables 1 and 3, there is a correlation between the binding of radiolabeled human proteins to the bacterial strains (Table 1) and the effect of human proteins on the partition of the bacterial strains in the dextran-PEG system (Table 3). The biological significance of the bacterial protein binding and subsequent changes of physicochemical surface properties of the bacteria is not yet clear. Such interactions might, however, contribute to interference with the entry of bacteria into the host and host cells and also to interference with host defense mechanisms.

In aqueous polymer phase systems it is possible to separate particles according to their surface properties by partition between the two immiscible phases and the interface (2). The polymers are gentle to the cell particles and to proteins (1). Phase partition has the further advantage that bacteria and protein can be partitioned together and therefore allow the specific protein-protein interactions to take place within the system. Only if there is an interaction between protein and the bacteria will the presence of protein change their distribution. Experiments performed in our laboratory have also documented the possible use of the polymer phase system for discovering unrecognized specific protein binding to bacterial cells.

The effect of bovine and murine proteins on the strains studied was completely in agreement with previous results on direct binding of radio-

TABLE 3. Effect of binding of human proteins to bacterial strains on partition in the dextran-PEG system^a

Bacterial strains	Serum	Human serum albumin	IgG	Fibrinogen
<i>Staphylococcus aureus</i>	↓↓	—	↓↓↓	↓
Group A <i>Streptococcus</i> , AW43	↑↑	↑	↑↑	↑↑
Group A <i>Streptococcus</i> , AM1	↑↑	—	↑↑	↑
Group G <i>Streptococcus</i> , G148	↓↓↓	↓↓↓	↓↓	—

^a One, two, and three arrows, respectively, denote low, medium, and high effect of protein on partition of bacterial cells in the phase systems selected for each individual bacterial strain. A dash represents no effect. The direction of the arrow denotes a change in partition in favor of the top phase (↑) or the bottom phase (↓) in the presence of protein as compared to the partition of bacteria alone.

labeled protein, with one exception. The slight but definite change in partition of the group G *Streptococcus* with bovine serum albumin and of the group A streptococci with human serum albumin has not been confirmed by direct binding studies and needs to be further investigated.

The change in distribution of bacteria after protein binding might be ascribed to the partition properties of the bound protein itself. However, the partition properties of bound protein could not be the sole factor influencing the distribution of bacteria, since the binding of one protein results in a change in partition in favor of the top phase of some strains (AW43 and AM1), but a change in partition in favor of the bottom phase of other strains (*S. aureus* and G148; Table 3). In spite of the lack of correlation between the partition behavior of protein alone and the observed change in partition of bacteria after specific protein binding, it is possible that bound protein expresses different physicochem-

ical properties as compared to protein in solution. As for immunoglobulin, there is an orientation of the bacteria-bound immunoglobulin molecules with their more hydrophilic Fab parts outward (16). A third mechanism mediating the observed surface changes might be that the binding of protein leads to a masking of bacterial cell surface properties. Receptor areas will be covered through the binding of protein to the bacterial cell surface and are no longer available to the polymers. In support of this, it is of particular interest that each individual bacterial strain shows the same change in partition after positive protein binding, whereas individual proteins have opposite effects on the partition of the different bacterial strains (Table 3).

Determination of surface characteristics of *S. typhimurium* has revealed that rough, nonvirulent bacteria are subject to hydrophobic and ionic interactions, whereas hydrophilicity and absence of charge are characteristic of smooth bacteria (22, 34). Therefore, it might be possible to predict the outcome of the interaction between protein-coated bacteria and host cells on the basis of results obtained in polymer two-phase systems. Experiments have been initiated to further characterize the change in surface properties of gram-positive cocci after specific binding of protein, with emphasis on susceptibility to ionic and hydrophobic interactions.

Extensive studies have been performed on the correlation between the physicochemical properties of bacteria and proneness to phagocytic engulfment (33, 36-38, 41, 42) as well as attachment to intestinal mucosal cells (27). From these investigations it is evident that the physicochemical cell surface properties of the bacteria play a crucial role in the interaction of bacteria with human cells. Many *in vitro* studies on adherence of streptococci to epithelial surfaces have been performed in physiological saline (4, 5, 10). Other authors have shown that there are surface differences between microorganisms grown *in vivo* and *in vitro* (31-33). The marked effects of specific protein binding to the bacteria on their surface properties as shown in our studies indicate that these types of interactions have to be taken into account in studies of virulence. Some low avid interactions between serum proteins and bacterial surfaces of more nonspecific type might also influence host-parasite interactions. However, in the present studies such low avid interactions did not influence the polymer phase partition of the investigated bacteria to any detectable extent.

Interference with host defense mechanisms after surface acquisition of host proteins has been demonstrated in experimental *Schistosoma mansoni* infection (6). This mammalian

parasite is capable of specific binding of blood group substances and histocompatibility antigens to its surface (8, 29). Receptors for IgG Fc and human β_2 -microglobulin have also been discovered on the surface of *S. mansoni* (14, 40). The existence of receptors for some types of human host proteins in such widely separated parasite species as schistosomes and streptococci seems to indicate that binding of host proteins might be a more widespread phenomenon in nature. Such binding might influence the outcome of host-parasite relationships as emphasized by the marked changes in surface properties of coated bacteria noted in our present studies. Their systematic elucidation will therefore provide a more solid basis for future virulence studies.

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LITERATURE CITED

1. Albertsson, P.-Å. 1970. Partition of cell particles and macromolecules in polymer two-phase systems. *Adv. Protein Chem.* 24:309-341.
2. Albertsson, P.-Å. 1971. Partition of cell particles and macromolecules. Wiley-Interscience, New York.
3. Albertsson, P.-Å. 1978. Partition between polymer phases. *J. Chromatogr.* 159:111-122.
4. Beachey, E. H. 1975. Binding of group A streptococci to human oral mucosal cells by lipoteichoic acid. *Trans. Assoc. Am. Physicians* 88:285-292.
5. Beachey, E. H., and I. Ofek. 1976. Epithelial cell binding of group A streptococci by lipoteichoic acid on fimbriae denuded of M protein. *J. Exp. Med.* 143:759-771.
6. Bloom, B. 1979. Games parasites play: how parasites evade immune surveillance. *Nature (London)* 279:21-26.
7. Christensen, P., and V.-A. Oxelius. 1975. A reaction between some streptococci and IgA myeloma proteins. *Acta Pathol. Microbiol. Scand.* 83:184-188.
8. Clegg, I. A., S. R. Smithers, and R. I. Terry. 1971. Acquisition of human antigens by *Schistosoma mansoni* during cultivation *in vitro*. *Nature (London)* 232:653-655.
9. Duthie, E. S. 1955. The action of fibrinogen on certain pathogenic cocci. *J. Gen. Microbiol.* 13:383-393.
10. Ellen, R. P., and R. I. Gibbons. 1972. M protein-associated adherence of *Streptococcus pyogenes* to epithelial surfaces: prerequisite for virulence. *Infect. Immun.* 5:826-830.
11. Forsgren, A., and J. Sjöquist. 1966. Protein A from *S. aureus*. I. Pseudo-immune reaction with human γ -globulin. *J. Immunol.* 97:822-827.
12. Harboe, M., and I. Fölling. 1974. Recognition of two distinct groups of human IgM and IgA based on different binding to staphylococci. *Scand. J. Immunol.* 3:471-482.
13. Kantor, F. S. 1965. Fibrinogen precipitation by streptococcal M protein. I. Identity of the reactants, and stoichiometry of the reaction. *J. Exp. Med.* 121:849-859.
14. Kemp, W. M., S. C. Merritt, M. S. Boguchi, J. C. Rosier, and J. R. Seed. 1977. Evidence of adsorption of heterospecific host immunoglobulin on the tegmen-

- tum of *Schistosoma mansoni*. J. Immunol. 119:1849-1854.
15. Kohler, W., and O. Prokop. 1978. Relationship between haptoglobin and *Streptococcus pyogenes* T4 antigens. Nature (London) 271:373.
 16. Kronvall, G. 1973. A surface component in group A, C and G streptococci with non-immune reactivity for immunoglobulin G. J. Immunol. 111:1401-1406.
 17. Kronvall, G., L. Björck, E. Myhre, and L. Wannamaker. 1979. Binding of aggregated β_2 -microglobulin, IgG, IgA and fibrinogen to group A, C and G streptococci with special reference to streptococcal M-protein. Proceedings of the VII International Symposium on Streptococcal Diseases.
 18. Kronvall, G., E. B. Myhre, L. Björck, and I. Berggård. 1978. Binding of aggregated human β_2 -microglobulin to surface protein structure in group A, C, and G streptococci. Infect. Immun. 22:136-142.
 19. Kronvall, G., C. Shönbeck, and E. Myhre. 1979. Fibrinogen binding structures detected in β -hemolytic streptococci group A, C and G are different from receptors for IgG and aggregated β_2 -microglobulin. Acta Pathol. Microbiol. Scand. Sect. B 87:303-310.
 20. Kronvall, G., A. Simmons, E. B. Myhre, and S. Jonsson. 1979. Specific adsorption of human serum albumin, immunoglobulin A, and immunoglobulin G with selected strains of group A and G streptococci. Infect. Immun. 25:1-10.
 21. Magnusson, K.-E., and G. Johansson. 1977. Probing the surface of *Salmonella typhimurium* and *Salmonella minnesota* SR and R bacteria by aqueous biphasic partitioning in systems containing hydrophobic and charged polymers. FEMS Microbiol. Lett. 2:225-228.
 22. Magnusson, K.-E., O. Stendahl, C. Tagesson, L. Edebo, and G. Johansson. 1977. The tendency of smooth and rough *Salmonella typhimurium* bacteria and lipopolysaccharide to hydrophobic and ionic interaction as studied in aqueous polymer two-phase systems. Acta Pathol. Microbiol. Scand. Sect. B 85:212-218.
 23. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of protein for immunologic studies. Int. Arch. Allergy 29:185-189.
 24. Much, H. 1908. Über eine Vorstufe des Fibrinfermentes in kulturen von *Staphylokokkus aureus*. Biochem. Z. 14:143-155.
 25. Myhre, E. B., and G. Kronvall. 1977. Heterogeneity of nonimmune immunoglobulin Fc reactivity among gram-positive cocci: description of three major types of receptors for human immunoglobulin G. Infect. Immun. 17:475-482.
 26. Myhre, E. B., and G. Kronvall. 1980. Demonstration of specific binding sites for human serum albumin in group C and G streptococci. Infect. Immun. 27:6-14.
 27. Perers, L., L. Andåker, L. Edebo, O. Stendahl, and C. Tagesson. 1977. Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. Acta Pathol. Microbiol. Scand. Sect. B 85:308-316.
 28. Rosa, U., G. A. Scassellati, F. Pennisi, N. Riccioni, P. Giagnoni, and R. Giordani. 1964. Labeling of human fibrinogen with ^{125}I by electrolytic iodination. Biochim. Biophys. Acta 86:519-526.
 29. Sher, A., B. F. Hall, and M. A. Vadas. 1978. Acquisition murine histocompatibility complex gene products by schistosomula of *Schistosoma mansoni*. J. Exp. Med. 148:46-57.
 30. Smith, H. 1964. Microbial behaviour in natural and artificial environments. Symp. Soc. Gen. Microbiol. 14:1-29.
 31. Smith, H. 1976. Survival of vegetative bacteria in animals. Symp. Soc. Gen. Microbiol. 26:299-326.
 32. Smith, H. 1977. Microbial surfaces in relation to pathogenicity. Bacteriol. 41:475-500.
 33. Stendahl, O., and L. Edebo. 1972. Phagocytosis of mutants of *Salmonella typhimurium* by rabbit polymorphonuclear cells. Acta Pathol. Microbiol. Scand. Sect. B 80:481-488.
 34. Stendahl, O., L. Edebo, K.-E. Magnusson, C. Tagesson, and S. Hjertén. 1977. Surface-charge characteristics of smooth and rough *Salmonella typhimurium* bacteria determined by aqueous two-phase partitioning and free zone electrophoresis. Acta Pathol. Microbiol. Scand. Sect. B 85:334-340.
 35. Stendahl, O., K.-E. Magnusson, C. Tagesson, R. Cunningham, and L. Edebo. 1973. Characterization of mutants of *Salmonella typhimurium* by counter-current distribution in an aqueous two-polymer phase system. Infect. Immun. 7:573-577.
 36. Stendahl, O., B. Normann, and L. Edebo. 1979. Influence of O and K antigens on the surface properties of *Escherichia coli* in relation to phagocytosis. Acta Pathol. Microbiol. Scand. Sect. B 87:85-91.
 37. Stendahl, O., C. Tagesson, and M. Edebo. 1973. Partition of *Salmonella typhimurium* in a two-polymer aqueous phase system in relation to liability to phagocytosis. Infect. Immun. 8:36-41.
 38. Stjernström, I., K.-E. Magnusson, O. Stendahl, and C. Tagesson. 1977. Liability to hydrophobic and charge interaction of smooth *Salmonella typhimurium* 395 MS sensitized with anti-MS immunoglobulin G and complement. Infect. Immun. 18:261-265.
 39. Tillet, W. S., and R. L. Garner. 1934. The agglutination of hemolytic streptococci by plasma and fibrinogen. A comparison of the phenomenon to serological reactions with the same organisms. Bull. Johns Hopkins Hosp. 54:145-156.
 40. Torpier, G., A. Capron, and M. A. Quaiissi. 1979. Receptor for IgG (Fc) and human β_2 -microglobulin on *S. mansoni* schistosomula. Nature 278:447-449.
 41. van Oss, C. J. 1978. Phagocytosis as a surface phenomenon. Annu. Rev. Microbiol. 32:19-39.
 42. van Oss, C. J., C. F. Gillman, and A. W. Neumann. 1975. Phagocytic engulfment and cell adhesiveness. Marcel Dekker, New York.

Isoelectric Points and Surface Hydrophobicity of Gram-Positive Cocci as Determined by Cross-Partition and Hydrophobic Affinity Partition in Aqueous Two-Phase Systems

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Thirty-nine streptococcal strains belonging to groups A, C, and G and 12 staphylococcal strains were investigated with respect to surface charge and hydrophobicity. Isoelectric points of the bacteria were determined by cross-partition experiments in dextran-polyethylene glycol two-phase systems containing charged polymers. The results obtained indicate that group A, C, and G streptococci have isoelectric points of $\text{pH } 3.75 \pm 0.15$ standard deviation. Staphylococci show an isoelectric point of around $\text{pH } 2$ and thereby differ markedly from the streptococci. Pretreatment of bacteria with human serum resulted in a significant change in the isoelectric points of streptococci. In a second series of experiments, an aqueous dextran-polyethylene glycol two-phase system containing polyethylene glycol palmitate or stearate was used to study the hydrophobic surface properties of the bacterial cells. The partition of the staphylococci was not influenced by the addition of up to 1% (wt/wt) polyethylene glycol palmitate or stearate, whereas the streptococci showed a large variation in affinity for polyethylene glycol-bound hydrophobic groups. The bacterial strains included in the study were also tested for uptake of human serum proteins. A positive correlation was found between the hydrophobic affinity of group A streptococci and the density of receptors for aggregated beta-2-microglobulin.

The interaction of bacteria with mammalian cells is of great importance in pathogenicity (31). Recently, surface properties of bacteria, such as charge and hydrophobicity, have received increased attention. Studies on *Salmonella typhimurium* have shown that the phagocytosis-sensitive R-mutants are liable to hydrophobic and ionic interactions, whereas the smooth phagocytosis-resistant strains have a hydrophilic and uncharged surface (22). Investigations with hydrophobic interaction chromatography suggest that the M-protein of *Streptococcus pyogenes* and protein A of *Staphylococcus aureus* contribute to the hydrophobic character of the bacterial cell surface (37, 38, 41). Specific binding of human proteins to receptors on gram-positive bacteria have been shown to change their physico-chemical surface properties (24).

Partition between two immiscible liquid phases composed of different aqueous polymer solutions has been used to investigate surface properties of *Salmonella typhimurium*, *Escherichia coli*, *Neisseria gonorrhoeae*, and various streptococcal and staphylococcal strains (5, 8, 19, 22, 24, 27, 28, 33-36). Such phase systems containing dextran and polyethylene glycol

(PEG) in water are highly useful for the separation and study of biological substances (2-4, 42). Particles, such as bacteria, distribute between the two phases and the interphase according to their surface properties, such as electrical charge and hydrophilic-hydrophobic character (4). To increase the selectivity of a polymer two-phase system, charged and hydrophobic groups have been covalently bound to the phase polymers (1, 10, 43, 44). With aqueous two-phase systems containing charged PEG, it is possible to perform a so-called cross-partition whereby the isoelectric points of the bacteria can be estimated (1, 2, 6, 9, 14). The hydrophobic surface properties of the bacteria were studied by hydrophobic affinity partition in two-phase systems containing PEG palmitate (P-PEG) or PEG stearate (St-PEG) (10, 20-22, 43, 44).

In the present investigation the isoelectric points of various group A, C, and G streptococci and staphylococci were determined by cross-partition. Pretreatment of bacteria with serum had a marked effect on the isoelectric point of the exposed bacterial surface and might have a bearing on the outcome of host-parasite interactions in vivo. The observed differences in hydro-

phobicity among the streptococci were correlated to the presence of specific receptors for human proteins.

MATERIALS AND METHODS

Bacterial strains. A total of 43 laboratory-maintained strains, 8 freshly isolated strains, and 8 heat-inactivated strains were included in the study. Laboratory-maintained strains were kindly supplied by D. Mirelman, Rehovot, Israel (one strain), by I. Rotta, Prague, Czechoslovakia (one strain), and by L. W. Wannamaker, Minneapolis, Minn. (six strains) or were obtained consecutively from clinical specimens sent to the Clinical Microbiology Laboratory, University Hospital, Lund, Sweden. The bacterial species and numbers of laboratory-maintained strains were as follows: *Staphylococcus aureus*, 6; *Staphylococcus epidermidis*, 2; *Staphylococcus saprophyticus*, 2; group A streptococci, 13; group C streptococci, 11; and group G streptococci, 10. All strains were kept at 4°C on blood agar plates before use. Todd-Hewitt broth cultures (Difco Laboratories, Detroit, Mich.) were incubated overnight at 37°C. Bacteria were harvested by centrifugation at 3,000 rpm for 15 min and washed twice in phosphate-buffered saline (PBS; 0.03 M sodium phosphate, 0.12 M NaCl, pH 7.2). The optical density at 620 nm was measured, and the bacterial concentration was calculated from a standard curve and adjusted to 10^9 organisms per ml. Heat-inactivated bacteria were prepared by heat treatment at 80°C for 5 min.

Radiolabeling of bacteria. Bacteria were labeled with ^{51}Cr (The Radiochemical Centre, Amersham, England). A 50- μCi portion of ^{51}Cr was added to 10^9 live bacteria in PBS and incubated at 37°C for 3 h. Heat-inactivated bacteria were labeled with 100 μCi of ^{51}Cr per 10^9 bacteria and incubated overnight. The bacteria were then deposited by centrifugation, washed four times in PBS, and suspended in sterile water to contain 5×10^8 bacteria per ml. Radioactivity in the last supernatant never exceeded 2% of that in the pellet. Radioactivity per bacterial cell was in the range of 10^{-3} cpm.

Human protein preparations. Human serum albumin, human polyclonal immunoglobulin (immunoglobulin G [IgG]), and human fibrinogen were purchased from Kabi AB (Stockholm, Sweden). IgA was prepared from a serum containing a high concentration of an IgA myeloma protein as described previously (25). Preparations of aggregated human beta-2-microglobulin were kindly supplied by L. Björck, Lund, Sweden. Human serum albumin, IgG, IgA, and aggregated beta-2-microglobulin were labeled with ^{125}I (The Radiochemical Centre) by using the chloramine-T method (23). Fibrinogen was radiolabeled by using the electrolytic method of Rosa et al. (29), as described by Harboe and Fölling (11).

Human protein-binding assay. Overnight cultures of bacterial strains in Todd-Hewitt broth were harvested and washed twice in PBS. The bacterial concentration was adjusted to 10^9 organisms per ml. The bacteria were suspended in PBS with 0.05% Tween 20. Binding studies were carried out in plastic tubes (70 by 12 mm; AB CERBO, Trollhättan, Sweden) by adding 2×10^8 bacteria (200- μl suspension containing 10^9 organisms per ml) to radiolabeled protein. The amounts used were 0.08 to 0.17 μg except for beta-2-microglobulin

(2.12 μg). After 1 h at room temperature, 2 ml of PBS containing Tween 20 was added to each tube, and the bacteria were centrifuged. The supernatants were removed, and the radioactivity of the bacterial pellets was measured in a gamma counter (LKB Rack Gamma, model 1270; Biotec, Stockholm, Sweden). The uptake of human serum proteins in percentage of total radioactivity added was calculated (Table 1). An uptake of <6% was considered negative. All uptake assays were performed in duplicate.

Polymer two-phase systems. PEG 4000 was obtained from Union Carbide, New York, N.Y., and Dextran T500 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Positively charged trimethylamino PEG (TMA-PEG), negatively charged PEG sulfonate (S-PEG), and P-PEG synthesized from PEG 4000 (15) were kindly supplied by G. Johansson, University of Lund, Lund, Sweden. St-PEG 4000 was purchased from Serva, Heidelberg, West Germany. The phase systems were prepared from stock solutions of dextran (20%), PEG 4000 (40%), TMA-PEG (10%), S-PEG (10%), P-PEG (2.5%), and St-PEG (2.5%). Salt and buffer stock solutions were made in concentrations 10 to 20 times higher than that of the final concentration in the phase system. All phase systems were prepared and all experiments were carried out at room temperature.

Cross-partition experiments. Single-tube partitions were performed in plastic tubes (70 by 12 mm; AB Cerbo) with 1.00-g phase systems. Two series of phase systems were prepared, one with positively charged TMA-PEG and one with negatively charged S-PEG. The compositions of the phase systems are given in Table 2 (phase systems A and B). Batch systems were prepared from stock solutions of dextran, PEG 4000, and TMA-PEG 4000 or S-PEG 4000. A 0.85-g sample was taken from the batch systems immediately after mixing, using a Cornwall pipette (Becton, Dickinson & Co., Rutherford, N.J.). Adding 50 μl of buffer solution and 100 μl of bacteria (5×10^7 bacteria) in sterile water gave the desired final concentration of all components. Sodium citrate-citric acid and citrate HCl buffers were used to obtain pH values between 3.0 and 7.2 and between 1.3 and 4.8, respectively. The phase systems were mixed thoroughly by inverting the tubes about 50 times. After 40 min of settling time, 100 μl of the top or bottom phase was withdrawn from each tube with a calibrated pipette (Oxford Laboratories, Foster City, Calif.). The radioactivity in each sample was measured in a gamma scintillation counter (LKB-Wallace 1280 Ultrascan; Biotec). The percentage of bacterial cells in the top phase (in some experiments in top and bottom phases) was calculated. The pH was measured on the residual phase system.

Hydrophobic affinity partition experiments. Single-tube partitions were performed with 1.00-g phase systems of the compositions given in Table 2 (phase systems C and D). A batch system was prepared from stock solutions of dextran, PEG 4000, sodium phosphate buffer, and sodium chloride. A 0.80-g sample was taken from the batch system immediately after mixing. Adding 100 μl of esterified PEG (P-PEG or St-PEG) or sterile water and 100 μl of bacterial suspension gave the final concentration of all components. The phases were mixed thoroughly and allowed to separate for 40 min. From the bottom phase or the top and bottom phases 100 μl was withdrawn, and deter-

TABLE 1. Binding of radiolabeled human proteins to bacterial strains^a

Bacterial strains	Uptake (% total radioactivity added to cells)				
	IgG	IgA	Beta-2-micro-globulin	Albumin	Fibrinogen
Group A streptococcal strains					
A1	24	7	19	6	65
A207	39	15	42	2	18
AM1	53	20	53	2	52
AM18	18	22	93	3	62
AM23	47	19	77	2	33
AM24	19	5	19	3	65
AM28	12	7	17	6	68
AR1	86	6	17	5	68
AW1	14	43	28	3	43
AW4	14	21	86	3	58
AW25	39	7	18	6	68
AW33	15	34	45	3	45
AW43	16	23	29	3	47
HA1	16	5	32	3	55
HA2	28	11	59	3	35
Group C and G streptococcal strains					
C16	82	7	3	30	51
C48	83	8	22	33	55
G34	86	11	4	44	51
G41	92	10	6	33	49
Staphylococcal strains					
Cowan 1	78	2	2	2	5
L603	5	3	4	3	4

^a Includes all investigated group A streptococci and representative strains of group C and G streptococci as well as staphylococci.

mination of the bacteria in the phases was made by measuring the radioactivity in a gamma scintillation counter. In the phase systems used for hydrophobic affinity partition experiments, the bacteria partitioned between bottom phase and interphase. For comparison of the relative hydrophobicities among the various bacterial strains, a hydrophobicity index (*C*) was calculated by using the following formula (see Fig. 5): $C = [\text{counts per minute in bottom phase in system with } 0.10\% \text{ (wt/wt) P-PEG} / \text{counts per minute in bottom phase in system without P-PEG}]$.

RESULTS

Isoelectric point of streptococci. The isoelectric point of 28 streptococcal strains was determined by cross-partition and included 16 laboratory-

maintained strains, 6 freshly isolated strains, and 6 heat-inactivated strains. The laboratory-maintained strains showed cross-points between pH 3.50 and 4.10, with a mean of 3.75 (Table 3). Figure 1 shows representative results from a cross-partition of four strains of streptococci. The freshly isolated streptococcal strains showed cross-points between pH 3.60 and 3.95 (mean, 3.74) and thereby did not differ from the laboratory-maintained strains. The cross-points of the heat-inactivated strains were in all instances lower than the corresponding live bacteria tested on the same occasion. Two bacterial strains (G148 and ARI) tested on five different occasions showed a standard deviation in cross-

TABLE 2. Composition of phase systems for single-tube partition

Phase system	% (wt/wt)						mM	
	Dextran T500	PEG 4000	TMA-PEG	S-PEG 4000	P-PEG 4000	St-PEG 4000	Buffer (see text)	Sodium phosphate buffer (pH 6.9)
A	6.8	4.8	2.0				2.5	
B	6.8	4.8		2.0			2.5	
C	6.1	6.1			0-0.5			5.0
D	6.1	6.1				0-0.5		5.0
								40.0
								40.0

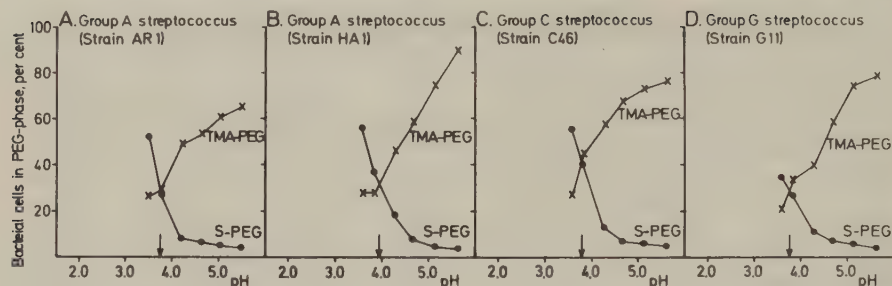


FIG. 1. Cross-partition of two group A streptococcal strains, AR1 and HA1 (A and B), one group C streptococcal strain, C46 (C), and one group G streptococcal strain, G11 (D). Symbols: $\times$, partition with TMA-PEG (positively charged); $\bullet$, phase systems with S-PEG (negatively charged). Arrows indicate cross-point values.

point values of not more than 0.11 and 0.025 pH units, respectively.

Isoelectric point of staphylococci. The isoelectric point of nine staphylococcal strains was determined by cross-partition and included five laboratory-maintained strains, two freshly isolated strains, and two heat-inactivated strains. Table three summarizes the mean cross-point value for the staphylococcal strains. For the laboratory-maintained strains and the freshly isolated strains, a cross-point below pH 2.2 was obtained (mean, pH 1.95). Figure 2 shows representative results from cross-partition of two staphylococcal strains. The cross-point values of the heat-inactivated strains were in both instances significantly higher than those for the corresponding live bacteria tested on the same occasion (mean, pH 2.65).

Effect of human serum on bacterial cross-partition. Five bacterial strains capable of binding different human serum proteins were preincubated with human serum before analysis. A 100- μ l portion of normal human serum, diluted 1:10 in PBS, was added to 8×10^8 bacterial organisms and incubated at room temperature for 30 min. The bacteria were then washed once in

PBS and suspended in sterile water. The isoelectric point of the bacteria pretreated with serum was determined by cross-partition and compared with the cross-point values obtained with the same bacteria pretreated with PBS alone as a control. The cross-partition of all four streptococcal strains studied was strongly influenced by pretreatment with human serum, resulting in less acidic cross-points (Table 4; Fig. 3). In contrast, pretreatment of *S. aureus* with human serum did not lead to any detectable change of isoelectric point by this method (Table 4).

Hydrophobic affinity partition. Relative surface hydrophobicity of 12 staphylococcal strains, 15 group A streptococci, 12 group C streptococci, and 12 group G streptococci was determined by hydrophobic affinity partition, using P-PEG (phase system C) and St-PEG (phase system D; Table 2). All bacteria studied were affected to the same extent by P-PEG and St-PEG.

The streptococci varied greatly in affinity for the PEG-bound groups. Most of the group A streptococci studied (11 of 15) gave results indicating a high affinity for P-PEG (Fig. 4A and 5). The remaining group A streptococci showed no

TABLE 3. Cross-point values of streptococcal and staphylococcal strains^a

Bacteria	No. of strains ^b	Cross-point (mean pH $\pm$ SD)
Group A streptococci	8	3.67 $\pm$ 0.15
Group C streptococci	7	3.80 $\pm$ 0.12
Group G streptococci	7	3.80 $\pm$ 0.16
Staphylococci	7	1.95 $\pm$ 0.16

^a The cross-point pH is taken as an estimate of the isoelectric point of the exposed bacterial surfaces.

^b Includes both laboratory-maintained and freshly isolated strains.

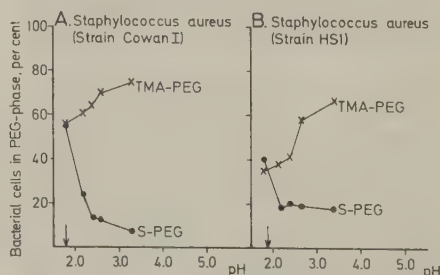


FIG. 2. Cross-partition of two *Staphylococcus aureus* strains. Symbols as in Fig. 1.

TABLE 4. Effect of human serum on cross-point values of bacteria

Bacterial strain	Cross-point (pH)	
	Cells pretreated with human serum	Cells untreated
Group A streptococci, ARI	4.80	3.75
Group A streptococci, AMI	4.20	3.50
Group C streptococci, C16	4.35	3.95
Group G streptococci, G148	4.80	3.90
<i>Staphylococcus aureus</i> Cowan I	<1.80 ^a	1.80

^a No cross-point was obtained within the pH range used in this experiment.

affinity for P-PEG in concentrations up to 0.25% (wt/wt). Among the group C and G streptococci there was a more even distribution among the strains with high, medium, and low affinity for P-PEG (Fig. 4B and 4C and 5). The partition of the staphylococci was not influenced by the addition of up to 1% (wt/wt) P-PEG or St-PEG (Fig. 5). Figure 4D shows the partition of three staphylococcal strains as a function of the concentration of P-PEG.

The freshly isolated streptococcal strains all had intermediate to high affinity for P-PEG, whereas the freshly isolated staphylococcal strains showed no affinity for P-PEG. Heat-inactivated streptococcal strains showed, when tested on the same occasion as the live bacteria, a lower hydrophobic affinity than the live bacteria in all instances. Hydrophobic affinity partition of heat-inactivated staphylococci was not altered compared with live bacteria.

DISCUSSION

In the present investigations the isoelectric points and surface hydrophobicity of gram-posi-

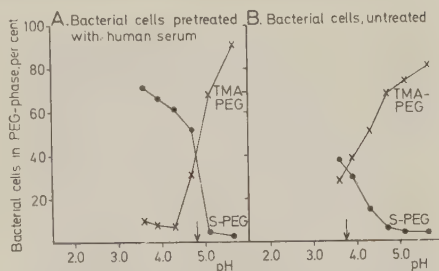


FIG. 3. Effect of normal human serum on cross-partition of one group A streptococcal strain (ARI). The bacteria were pretreated with human serum (A) or with PBS as a control (B) before cross-partition experiments. Symbols as in Fig. 1.

tive cocci have been determined by cross-partition and hydrophobic affinity partition in aqueous two-phase systems. Phase partition provides a useful tool for the study of surface charge and hydrophobicity in particles such as bacteria (3, 5, 8, 19–22, 24, 27, 33–36). Isoelectric points of the bacteria were determined by cross-partition experiments with dextran-PEG two-phase systems containing charged polymers (6, 9; Table 2). The results obtained indicate an isoelectric point for streptococci of about pH 3.75 (Fig. 1; Table 3). The staphylococci showed an isoelectric point of around pH 2, thereby differing markedly from the streptococci (Fig. 2; Table 3). Results from cross-partition of the ribitol teichoic acidless mutant 52 A5 (kindly supplied by D. Mirelman, Rehovot, Israel) indicated an isoelectric point of <pH 2. This observation suggests that teichoic acid makes only a minor contribution to the highly acidic cross-point of staphylococci.

The surface hydrophobicity of gram-positive cocci was assessed by determining the affinity

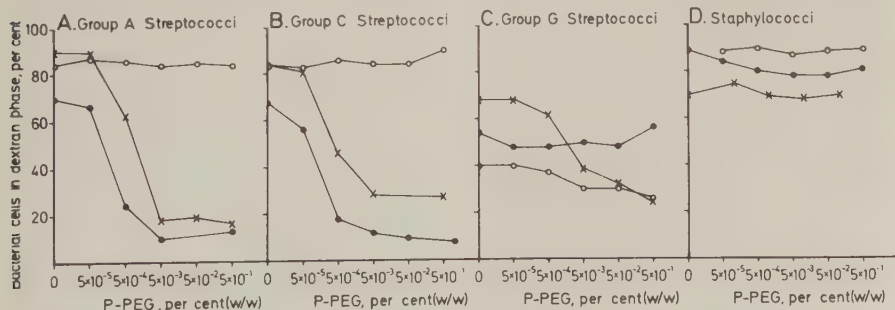


FIG. 4. Hydrophobic affinity partition in a dextran-PEG phase system at different P-PEG concentrations. Symbols: (A) ×, group A streptococcal strains AM23; ●, AM1; ○, AM28. (B) ×, Group C streptococcal strains C48; ●, C42; ○, C16. (C) ×, Group G streptococcal strains G41; ●, G34; ○, G51. (D) ×, Staphylococcal strains DO4975; ●, S48; ○, L602.

for PEG-bound hydrophobic groups. Two groups with different hydrophobic reactivities were identified among the group A streptococci. Most group A streptococci tested showed a high hydrophobic affinity, whereas partition of the remaining group A streptococci was not influenced by P-PEG (Fig. 5). Among the group C and G streptococci, there were several strains with intermediate reactivities giving a wider distribution of hydrophobic affinity (Fig. 5). The partition of the staphylococcal strains was not influenced by the addition of up to 1% (wt/wt) P-PEG or St-PEG (Fig. 4D and 5). *Staphylococcus saprophyticus* and *Staphylococcus epidermidis* are known to have a poor hydrophobic interaction liability (8). In our investigation this was also shown to be true for all *S. aureus* strains studied. It might be a general phenomenon that when a bacterial surface is highly negatively charged, it also shows a low hydrophobic affinity even if hydrophobic sites are present. The large number of negative charges might prevent the interaction of PEG-bound palmitoyl groups with hydrophobic structures on the bacterial surface.

An increasing number of specific interactions between surface structures of streptococci and human proteins have recently been discovered (17; G. Kronvall, L. Björk, H. Mörner, E. Myhre, and K. Widebäck, in S. E. Holm and P. Christensen [ed.], *Basic Concepts of Streptococci and Streptococcal Diseases*, in press). The bacterial strains included in this study were tested for uptake of human serum proteins (Table 1). A positive correlation was found between the hydrophobic affinity of group A streptococci and the density of specific receptors for aggregated beta-2-microglobulin (Table 1; Fig. 5). All group A streptococcal strains with low hydrophobic affinity showed a low uptake of radiolabeled aggregated beta-2-microglobulin (mean uptake, 17.5%), whereas the strains with high hydrophobic affinity showed a medium or high uptake of aggregated beta-2-microglobulin (mean uptake, 56%). It has been suggested that M-protein carries a receptor for aggregated beta-2-microglobulin (7). The results obtained indicate a relationship between M-protein and the hydrophobic properties of group A streptococcal cells which has previously been suggested by Tylewska et al. (37). The surface hydrophobicity of streptococci did not correlate with the presence of surface receptors for other human proteins tested (e.g., albumin, IgG, IgA, and fibrinogen).

We have previously described marked changes of surface characteristics of gram-positive cocci after specific binding of human proteins (24). Pretreatment of bacteria with human serum leads to an increase of the isoelectric

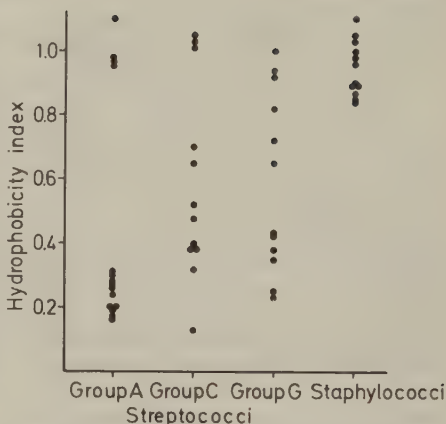


FIG. 5. Hydrophobicity index (C) of 50 human strains of group A, C, and G streptococci and staphylococci as calculated from the formula: $C = (\text{counts per minute in bottom phase in system with } 0.10\% \text{ P-PEG}) / (\text{counts per minute in bottom phase in system without P-PEG})$. The group A streptococci with low hydrophobic affinity include strains A1, AM28, AR1, and AW25.

points of the streptococci investigated, whereas no such increase could be observed with the *S. aureus* strain (Fig. 3; Table 4). We have previously suggested that the binding of protein to bacteria leads to a masking of the bacterial cell surface properties (24). Experiments have been initiated to further characterize the change in surface charge of gram-positive cocci after specific binding of various human serum proteins.

An aqueous dextran-PEG two-phase system containing P-PEG or St-PEG was used for hydrophobic affinity partition (phase systems C and D, Table 2). These phase systems have an interfacial potential close to zero; therefore, the bacteria distribute according to the ratio between hydrophilic and hydrophobic sites exposed on their surface. Most of the bacteria partitioned into the bottom phase in the absence of esterified PEG. The main change observed when the concentration of P-PEG or St-PEG was increased was the transfer of bacterial cells from the bottom phase into the interphase. The bacteria studied were affected to a similar extent by P-PEG and St-PEG.

No single method can adequately describe surface hydrophobicity, and there is not always a correlation between hydrophobic affinity partition and other methods of assessing surface hydrophobicity. A possible explanation is that hydrophobic affinity partition measures an overall quantitative expression of the hydrophobic and hydrophilic sites exposed on the bacterial

surface whereas with other methods (hydrophobic interaction chromatography, binding of hydrocarbons and fatty acids to cells; 16, 30, 31, 37–39, 41) a few strongly hydrophobic sites on the bacterial surface will allow the bacteria to adsorb to the hydrophobic ligand.

Cross-partition has previously been used for the determination of the isoelectric points of proteins, mitochondrial membranes, spinach thylakoid membranes, and rat liver peroxisomes (4, 6, 9, 14). Two curves were obtained when the percentage of bacteria in the top phase was plotted against the pH (Fig. 1). The obtained cross-point of the curves was taken as the approximate isoelectric point of the bacteria. In the present investigation the bacteria were grown in a defined medium overnight. Variations of the electrophoretic mobility of streptococci harvested during different growth phases or prepared in different growth media have been reported (12, 13). The possible effect of different growth media and growth conditions on the surface properties of the bacteria have not yet been studied in polymer two-phase systems.

Several reports indicate that the surface charge of either the mammalian cells or the bacteria plays an important role in their mutual interaction (26, 33, 36, 40). Gram-positive cocci are negatively charged at neutral pH due to an excess of carboxyl and phosphate groups. In addition, some bacteria contain highly negatively charged teichoic and teichuronic acids. Most mammalian cells also carry a net negative charge which could give rise to a net repulsive force between bacteria and mammalian cells. Previous attempts have been made to measure the isoelectric point of bacteria. Electrokinetic studies of different strains of *Streptococcus pyogenes* indicate an isoelectric point of between pH 4.3 and 4.7 (12). The isoelectric point of *Streptococcus sanguis* has been determined by isoelectric focusing and was found to be pH 5.3 (18). Preliminary results from isoelectric focusing of M-protein-positive and M-protein-negative strains of *Streptococcus pyogenes* suggest isoelectric points of pH 5.8 and 6.0, respectively (37). M-proteins of streptococci play a major role in the pathogenicity of these bacteria. It appears that the presence of M-protein does not lead to any major change in surface charge, whereas the indicated hydrophobic properties of M-protein might be of importance in the interaction between vertebrate cells and *Streptococcus pyogenes*.

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LITERATURE CITED

- Åkerlund, H.-E., B. Andersson, A. Persson, and P.-Å. Albertsson. 1979. Isoelectric points of spinach thylakoid membrane surfaces as determined by cross-partition. *Biochim. Biophys. Acta* 552:238–246.
- Albertsson, P.-Å. 1970. Partition of cell particles and macromolecules in polymer two-phase systems. *Adv. Protein Chem.* 24:309–341.
- Albertsson, P.-Å. 1971. Partition of cell particles and macromolecules. Wiley-Interscience, New York.
- Albertsson, P.-Å. 1978. Partition between polymer phases. *J. Chromatogr.* 159:111–122.
- Albertsson, P.-Å., and G. D. Baird. 1962. Counter-current distribution of cells. *Exp. Cell Res.* 28:296–322.
- Albertsson, P.-Å., S. Sasakawa, and H. Walter. 1970. Cross-partition and isoelectric points of proteins. *Nature (London)* 228:1329–1330.
- Björck, L., S. K. Tylewska, T. Wadström, and G. Kronvall. 1981. Beta-2-microglobulin is bound to streptococcal M protein. *Scand. J. Immunol.* 13:391–394.
- Colleen, S., B. Hovelius, A. Wieslander, and P.-Å. Mårdh. 1979. Surface properties of *Staphylococcus saprophyticus* and *Staphylococcus epidermidis* as studied by adherence test and two-polymer aqueous phase system. *Acta Pathol. Microbiol. Scand. Sect. B* 87:321–328.
- Ericson, I. 1974. Determination of the isoelectric point of rat liver mitochondria by cross-partition. *Biochim. Biophys. Acta* 356:100–107.
- Eriksson, E., P.-Å. Albertsson, and G. Johansson. 1976. Hydrophobic surface properties of erythrocytes studied by affinity partition in aqueous two-phase systems. *Mol. Cell. Biochem.* 10:123–128.
- Harboe, M., and I. Fölling. 1974. Recognition of two distinct groups of human IgM and IgA based on different binding to *Staphylococci*. *Scand. J. Immunol.* 3:471–482.
- Hill, M. J., A. M. James, and W. R. Maxted. 1963. Some physical investigations of the behaviour of bacterial surfaces. VIII. Studies on the capsular material of *Streptococcus pyogenes*. *Biochim. Biophys. Acta* 66:264–274.
- Hill, M. J., A. M. James, and W. R. Maxted. 1963. Some physical investigations of the behaviour of bacterial surfaces. X. The occurrence of lipid in the streptococcal cell wall. *Biochim. Biophys. Acta* 75:414–424.
- Horie, S., H. Ishii, H. Nakazawa, T. Suga, and H. Orii. 1979. Determination of the cross-points of rat liver peroxisomes, peroxisomal core and the core components by cross-partition. *Biochim. Biophys. Acta* 585:435–443.
- Johansson, G. 1970. Studies on aqueous dextran-poly (ethylene glycol) two-phase systems containing charged poly (ethylene glycol). 1. Partition of albumins. *Biochim. Biophys. Acta* 222:381–389.
- Käppell, O., and A. Fiechter. 1976. The mode of interaction between the substrate and cell surface of the hydrocarbon-utilizing yeast *Candida tropicalis*. *Biotechnol. Bioeng.* 18:976–974.
- Kronvall, G., A. Simmons, E. B. Myhre, and S. Jonsson. 1979. Specific absorption of human serum albumin, immunoglobulin A, and immunoglobulin G with selected strains of group A and G streptococci. *Infect. Immun.* 25:1–10.
- Longton, R. W., I. S. Cole, and P. F. Quinn. 1975. Isoelectric focusing of bacteria: species location within an isoelectric focusing column by surface charge. *Arch. Oral Biol.* 20:103–106.
- Magnusson, K.-E., and G. Johansson. 1977. Probing the surface of *Salmonella typhimurium* and *Salmonella minnesota* S R and R bacteria by aqueous biphasic partitioning in systems containing hydrophobic and charged polymers. *FEMS Microbiol. Lett.* 2:225–228.
- Magnusson, K.-E., E. Kihlström, L. Norlander, A. Norqvist, J. Davies, and S. Normark. 1979. Effect of colony type and pH on surface charge and hydrophobicity of *Neisseria gonorrhoeae*. *Infect. Immun.* 26:379–401.
- Magnusson, K.-E., E. Kihlström, A. Norqvist, J. Davies,

- and S. Normark. 1979. Effect of iron on surface charge and hydrophobicity of *Neisseria gonorrhoeae*. *Infect. Immun.* 26:402-407.
22. Magnusson, K.-E., O. Stendahl, C. Tagesson, L. Edebo, and G. Johansson. 1977. The tendency of smooth and rough *Salmonella typhimurium* bacteria and lipopolysaccharide to hydrophobic and ionic interaction as studied in aqueous polymer two-phase systems. *Acta Pathol. Microbiol. Scand. Sect. B* 85:212-218.
 23. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of protein for immunologic studies. *Int. Arch. Allergy* 29:185-189.
 24. Mörner, H., E. Myhre, L. Björck, and G. Kronvall. 1980. Effect of specific binding of human albumin, fibrinogen, and immunoglobulin G on surface characteristics of bacterial strains as revealed by partition experiments in polymer phase systems. *Infect. Immun.* 29:879-885.
 25. Myhre, E. B., and G. Kronvall. 1979. Immunoglobulin binding to group A, C and G streptococci, p. 76-78. *In* M. T. Parker (ed.), *Pathogenic streptococci*. Reedbooks Ltd., Chertsey.
 26. Nagura, H., J. Asai, Y. Katsumata, and K. Kojima. 1973. Role of electric surface charge of cell membrane in phagocytosis. *Acta Pathol. Jpn.* 23:279-290.
 27. Perers, L., L. Andåker, L. Edebo, O. Stendahl, and C. Tagesson. 1977. Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. *Acta Pathol. Microbiol. Scand. Sect. B* 85:308-316.
 28. Pestka, S., H. Walter, and L. G. Wayne. 1977. Altered surface properties of *Escherichia coli* associated with a specific amino acid change in the S12 ribosomal protein of streptomycin-resistant mutants. *Antimicrob. Agents Chemother.* 11:978-983.
 29. Rosa, U., G. A. Scassellati, F. Pennisi, N. Riccioni, P. Giagnoni, and R. Giordani. 1964. Labelling of human fibrinogen with 131-I by electrolytic iodination. *Biochim. Biophys. Acta* 86:519-526.
 30. Rosenberg, M., D. Gutnick, and E. Rosenberg. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.* 9:29-33.
 31. Smith, H. 1977. Microbial surfaces in relation to pathogenicity. *Bacteriol. Rev.* 41:475-500.
 32. Smyth, C. I., P. Jonsson, E. Olsson, O. Söderlind, J. Rosengren, S. Hjerten, and T. Wadström. 1978. Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. *Infect. Immun.* 22:462-472.
 33. Stendahl, O., L. Edebo, K.-E. Magnusson, C. Tagesson, and S. Hjerten. 1977. Surface-charge characteristics of smooth and rough *Salmonella typhimurium* bacteria determined by aqueous two-phase partitioning and free zone electrophoresis. *Acta Pathol. Microbiol. Scand. Sect. B* 85:334-340.
 34. Stendahl, O., K.-E. Magnusson, C. Tagesson, R. Cunningham, and L. Edebo. 1973. Characterization of mutants of *Salmonella typhimurium* by counter-current distribution in an aqueous two-polymer phase system. *Infect. Immun.* 7:573-577.
 35. Stendahl, O., B. Normann, and L. Edebo. 1979. Influence of O and K antigens on the surface properties of *Escherichia coli* in relation to phagocytosis. *Acta Pathol. Microbiol. Scand. Sect. B* 87:85-91.
 36. Stendahl, O., C. Tagesson, and M. Edebo. 1973. Partition of *Salmonella typhimurium* in a two-polymer aqueous phase system in relation to liability to phagocytosis. *Infect. Immun.* 8:36-41.
 37. Tylewska, S., S. Hjerten, and T. Wadström. 1979. Contribution of M protein to the hydrophobic surface properties of *Streptococcus pyogenes*. *FEMS Microbiol. Lett.* 6:249-253.
 38. Tylewska, S. K., S. Hjerten, and T. Wadström. 1980. Hydrophobic properties of *Streptococcus pyogenes* related to M protein: decrease of surface hydrophobicity and epithelial cell binding by antibiotics, p. 788. *In* J. D. Nelson and C. Grassi (ed.), *Current chemotherapy and infectious diseases*, vol. 2. American Society for Microbiology, Washington, D.C.
 39. Tylewska, S. K., T. Wadström, and S. Hjerten. 1980. The effect of subinhibitory concentrations of penicillin and rifampicin on bacterial cell surface hydrophobicity and on binding to pharyngeal cells. *Antimicrob. Chemother.* 6:292-294.
 40. Van Oss, C. J. 1978. Phagocytosis as a surface phenomenon. *Annu. Rev. Microbiol.* 32:19-39.
 41. Wadström, T., S. Hjerten, P. Jonsson, and S. Tylewska. 1981. Hydrophobic surface properties of *Staphylococcus aureus*, *Staphylococcus saprophyticus*, and *Streptococcus pyogenes*: a comparative study, p. 263. *In* J. J. Jelszewicz (ed.), *Staphylococci and staphylococcal infection*. Gustav Fischer Verlag, Stuttgart.
 42. Walter, H. 1977. Partition of cells in two-polymer aqueous phases: a surface affinity method for cell separation, p. 307. *In* N. Catsimopoulos (ed.), *Methods of cell separation*. Plenum Publishing Corp., New York.
 43. Walter, H., E. J. Krob, and R. Tung. 1976. Hydrophobic affinity partition in aqueous two-phase systems of erythrocytes from different species. *Exp. Cell Res.* 102:14-24.
 44. Westrin, H., P.-Å. Albertsson, and G. Johansson. 1976. Hydrophobic affinity partition of spinach chloroplasts in aqueous two-phase systems. *Biochim. Biophys. Acta* 436:696-706.



Lipoteichoic Acid Is the Major Cell Wall Component Responsible for Surface Hydrophobicity of Group A Streptococci

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The contribution of lipoteichoic acid (LTA) to the hydrophobic surface properties of group A streptococci was investigated in aqueous dextran-polyethylene glycol two-phase systems. Enzymatic digestions were performed to characterize the hydrophobic surface structure. The results obtained indicated that LTA is a major factor responsible for the hydrophobic character of the cell surface of group A streptococci. This was further supported by the similarity of partition in polymer two-phase systems between whole group A streptococci and tritiated LTA extracted from a group A streptococcal strain. Surface LTA was also determined on intact organisms by a new method measuring the adsorption of antibodies to LTA to the bacterial surface. A correlation was found between the content of surface LTA and the hydrophobicity of the group A streptococci. We conclude that surface-associated LTA is the major factor determining surface hydrophobicity of group A streptococci.

Surface properties of bacteria are important in conferring or modulating adherence properties of the bacteria to host cells (20, 28, 32-34, 38, 39). The surface properties of a microorganism will therefore determine the outcome of a host-parasite interaction (31). Adherence of group A streptococci to epithelial surfaces is recognized as a prerequisite for the virulence of these organisms (9). Previous studies have demonstrated that the hydrophobic character of bacteria plays a central role in their interaction with mammalian cells (20, 28, 32, 38, 39). Group A streptococci are known to have a strong tendency to hydrophobic interaction (35). A relationship between M protein and the hydrophobic properties of these bacteria has previously been suggested (24, 35).

Lipoteichoic acid (LTA), another cell wall component of group A streptococci, has been suggested to play a central role in the pathogenesis of streptococcal infections (18, 46). LTA is an amphiphatic molecule composed of 1,3-phosphodiester-linked glycerophosphate and a small lipid moiety (46). Teichoic acids are exposed on the surface of intact bacteria (8, 21, 27, 37). Wicken and Knox have proposed that LTA may be transient within the cell wall (46). The hydrophobic lipid portion of LTA influences the immunogenicity of teichoic acid (45) and is essential for the binding of teichoic acids to cell membranes (7, 26).

In the present investigations, surface structures of group A streptococci responsible for

hydrophobicity were further characterized. Partition in aqueous polyethylene glycol (PEG)-dextran two-phase systems containing hydrophobic groups covalently bound to PEG was used to study the hydrophobic surface properties of the bacterial cells (1, 2, 11, 24, 40, 41, 43). LTA extracted from group A streptococci showed a hydrophobic interaction liability similar to that of whole organisms. The hydrophobic affinity of group A streptococci as revealed by partition in polymer two-phase systems correlated to the content of surface LTA. The results indicated that LTA rather than M protein contributes to the hydrophobic affinity of group A streptococci.

MATERIALS AND METHODS

Bacterial strains. A total of 56 bacterial strains were included in the study. The bacterial species and number of strains were as follows: group A streptococci, 34; group C streptococci, 5; group G streptococci, 6; *Klebsiella pneumoniae*, 3; *Pseudomonas aeruginosa*, 4; and *Escherichia coli*, 4. The strains were kindly provided by J. Rotta, Prague, Czechoslovakia (28 strains), and L. W. Wannamaker, Minneapolis, Minn. (2 strains), or were obtained consecutively from clinical specimens sent to the Clinical Microbiology Laboratory, University Hospital, Lund, Sweden. Strains were stored at -90°C in Todd-Hewitt broth supplemented with fetal calf serum and on blood agar plates at 4°C. Bacterial strains were inoculated in Todd-Hewitt broth and incubated for 15 to 17.5 h at 37°C. Bacteria were harvested by centrifugation at 3,500 rpm for 10 min and washed twice in phosphate-buffered

TABLE 1. Effects of enzymatic treatment and heat treatment of group A streptococci on the partition of the bacteria in polymer two-phase systems

Bacterial strain	PBS control	Trypsin ^b	CP ^a		Papain ^d	Hyaluronidase ^e	Heat ^f
			pH 4.5	pH 5.8			
AW4	65	14	40	72	24	72	62
AM1	48	10	26	42	17	62	52
AM18	75	12	28	72	26	92	75
AM23	78	16	35	78	26	77	81

^a See the text.^b A total of 150 µg of trypsin per ml, pH 7.2.^c A total of 100 µg of pepsin per ml.^d A total of 200 µg of papain per ml, pH 8.0.^e A total of 50 U of hyaluronidase per ml, pH 7.2.^f A temperature of 100°C for 10 min.

saline (PBS; 0.03 M sodium phosphate, 0.12 M NaCl, pH 7.2). The optical density at 620 nm was measured, and the bacterial concentration was calculated from a standard curve and adjusted to 10^9 organisms per ml.

Radiolabeling of bacteria. For bacterial radiolabeling, [³H]glycerol, [³H]thymidine, ⁵¹Cr (sodium chromate), and ¹²⁵I (sodium iodide) (Radiochemical Centre, Amersham, England) were used. Bacterial labeling with ¹²⁵I was performed by the following procedure. A total of 10^9 bacterial organisms were suspended in 200 µl of PBS. Then 15 µCi of ¹²⁵I, 5 µl of 0.015% hydrogen peroxide in PBS, and 5 µl of lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.) were added to the cell suspension and incubated at room temperature for 10 min. The bacteria were deposited by centrifugation, washed four times in ice-cold PBS, and suspended in PBS to 5.0×10^8 organisms per ml. Alternative labeling of bacteria with ⁵¹Cr

was performed as previously described (24). Internal labeling with [³H]thymidine was performed by growing bacteria in Todd-Hewitt broth containing 2 µCi of [³H]thymidine per ml (specific activity, 25 Ci/mmol; Radiochemical Centre). Cells were harvested, washed four times, and suspended in PBS to 10^9 organisms per ml. LTA was radiolabeled by growing the bacteria in Todd-Hewitt broth supplemented with 1.5 µCi of [³H]glycerol (specific activity, 500 mCi/mmol) per ml.

Enzymatic digestion and heat treatment of group A streptococci. Bacteria were treated with trypsin (Sigma Chemical Co.; catalog no. T-8253) by suspending 5.0×10^8 organisms in 0.5 ml of PBS containing 150 µg of trypsin per ml. In some experiments, increasing amounts of trypsin were added to the cells. After 45 min of incubation at 37°C, the tryptic activity was stopped by the addition of 0.5 mg of trypsin inhibitor (Sigma Chemical Co.; catalog no. T-9128) and by subsequent washings in PBS. Organisms were treated with pepsin (Sigma Chemical Co.; catalog no. P-7012) by washing them once in 0.1 M acetate buffer, pH 4.5 or in 0.1 M phosphate buffer, pH 5.8, and resuspending 5.0×10^8 bacteria in 0.5 ml of the respective buffer containing 100 µg of pepsin per ml. The mixture was incubated at 37°C for 30 min, and the reaction was terminated by the addition of 100 µl of 1 M Tris base. Treatment with papain (Sigma Chemical Co.; catalog no. P-9886) was performed by resuspending 5.0×10^8 bacteria in 0.5 ml of 0.1 M Tris base–0.02 M cysteine (pH 8.0) containing 200 µg of papain per ml. The mixture was incubated at 37°C for 45 min. The streptococci were also treated with hyaluronidase (Sigma Chemical Co.; catalog no. H-3506) by resuspending 5.0×10^8 bacteria in 0.5 ml of PBS containing 50 U of hyaluronidase per ml followed by incubation at 37°C for 60 min. Heat treatment of streptococci suspended in PBS was performed at 100°C for 10 min. After enzymatic and heat treatment, the bacteria were washed twice in ice-cold PBS and resuspended in appropriate solutions for use in polymer two-phase systems or LTA assays.

Polymer two-phase system. PEG 4000 was obtained from Union Carbide Corp., New York, N.Y., and dextran T500 (lot no. FD 16027) from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden. Stearate PEG (St-PEG) was purchased from Serva, Heidelberg, Federal

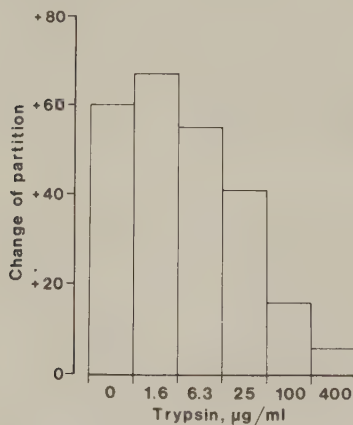


FIG. 1. Effect of trypsin digestion of a group A streptococcus (strain AM23) on the CP in polymer two-phase systems with and without 0.25% St-PEG. Bacteria were pretreated with various concentrations of trypsin as indicated. For phase composition and calculation of CP see the text.

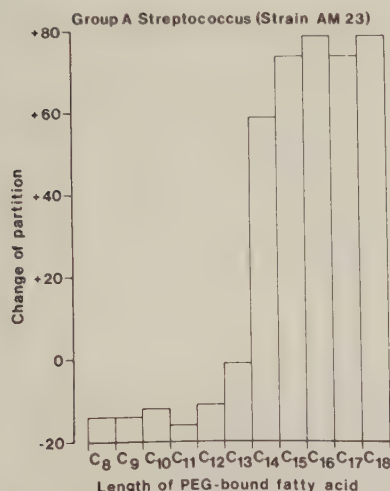


FIG. 2. Effect of PEG-bound fatty acids of various carbon chain lengths on the partition of a group A streptococcus (strain AM23) in polymer two-phase systems. PEG esters (0.5%) were used. For phase composition and calculation of CP see the text.

Republic of Germany. PEG esters (C_8 to C_{18} fatty acid esters of PEG 6000) were prepared with dicyclohexylcarbodiimide mainly according to Johansson (15), but without copper(I) iodide as a catalyst. Two-phase systems were prepared from stock solutions of dextran (20%), PEG 4000 (40%), and PEG esters (2.5 to 5.0%). Salt and buffer stock solutions were made up to concentrations 10 to 20 times higher than that of the final concentration in the phase system.

Hydrophobic affinity partition experiments. Single-tube partitions were performed in plastic tubes (70 by 11 mm; A/S NUNC, Roskilde, Denmark) with 1.00-g-weight phase systems. The phase systems were composed of 6.1% (wt/wt) dextran, 6.1% (wt/wt) PEG, 5 mM sodium phosphate buffer (pH 6.9), 40 mM NaCl, and 0 to 0.5% (wt/wt) PEG esters. A batch system was prepared from stock solutions of dextran, PEG 4000, sodium phosphate buffer, and sodium chloride. A 0.80-g sample was taken from the batch system immediately after mixing. Adding 100 μ l of esterified PEG solution or sterile water and 100 μ l of bacteria in sterile water gave the desired final concentration of all components. The phase systems were mixed thoroughly by inverting the tubes about 50 times. After a 40-min settling time, 100 μ l of the top and bottom phases was withdrawn from each tube with a calibrated pipette (Oxford Laboratories, Foster City, Calif.). The radioactivity in each sample was measured in a gamma counter (LKB-Wallac 1270 Rackgamma; LKB Sverige AB, Bromma, Sweden). In experiments with beta emitters, the samples were collected on glass micro-fiber filters (2.5 cm; Whatman Ltd., Maidstone, England). Dried filters were placed in scintillation vials, and 7.5 ml of scintillation fluid [5 g of PPO (2,5-diphenyloxazole) and 0.1 g of dimethyl 1,4-bis-(5-phenyloxazolyl)benzene (Packard Instrument Co.,

Inc., Rockville, Md.) dissolved in 1 liter of toluene (E. Merck AG, Darmstadt, Germany)] was added to each vial. The samples were counted in an Intertechnique SL 4000 scintillation counter (Plaisir, France). Phase systems were prepared, and experiments were carried out at room temperature. All experiments were performed in duplicate.

Presentation of partition data. The percent bacterial cells in the top and bottom phases was calculated from the radioactivity measured. The results are presented in the present studies as change of partition (CP) as described by Magnusson et al. (19). CP was calculated using the following formula: $CP = (T_{F-PEG} - B_{F-PEG}) - (T_{basal} - B_{basal})$ where T is the percent bacteria in the PEG-rich top phase and B is the percent bacteria in the dextran-rich bottom phase. F-PEG indicates the phase system containing PEG-bound fatty acid, and basal indicates the phase system without hydrophobic groups. The value of CP can vary in the range 0 to 200 and is positive when hydrophobic partitioning (i.e., transfer from the lower phase to the interface or from the interface to the upper phase) is involved. A negative value of CP means that the cells are excluded from the upper phase when the fatty acid group is introduced.

LTA assay. A rabbit antiserum raised against *Lactobacillus casei* LTA (kindly provided by K. W. Knox, Sydney, Australia) was used for determining cell wall LTAs on whole organisms. Staphylococcal protein A

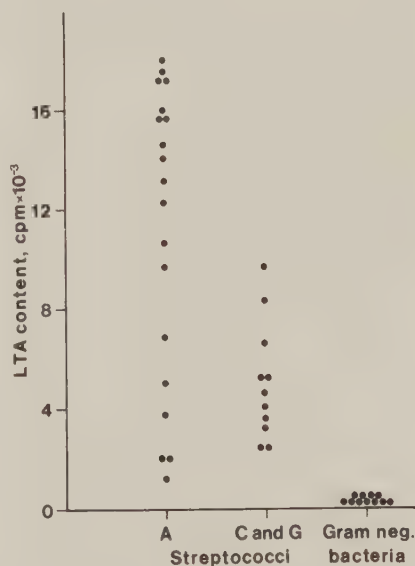


FIG. 3. Determination of surface LTA in bacterial strains. Group A, C, and G streptococci and gram-negative bacteria were labeled with antibodies to LTA and treated with 125 I-labeled protein A. The data are presented as corrected counts per minute (counts per minute with antibodies to LTA minus counts per minute with control consisting of normal rabbit serum).

TABLE 2. Effects of enzymatic treatment of group A streptococci on the binding of antibodies to LTA to their surfaces

Bacterial strain	PBS control	Trypsin ^a	Antibody binding (cpm $\times 10^3$)			
			Pepsin ^b		Papain ^c	Hyaluronidase ^d
			pH 4.5	pH 5.8		
AW4	7.1	1.4	4.5	5.5	4.6	6.1
AM1	9.6	2.0	4.2	7.0	4.1	8.9
AM18	7.9	1.7	3.8	6.3	3.5	6.7
AM23	9.2	1.3	2.5	9.5	2.6	9.4

^a A total of 150 μ g of trypsin per ml, pH 7.2.^b A total of 100 μ g of pepsin per ml.^c A total of 200 μ g of papain per ml, pH 8.0.^d A total of 50 U of hyaluronidase per ml, pH 7.2.

(Pharmacia Fine Chemicals) was labeled with 125 I by the chloramine-T method (22) and used as an anti-antibody reagent (12). The LTA assay was performed in duplicate in plastic tubes (70 by 12 mm; AB Cerbo, Trollhättan, Sweden). A 50- μ l amount of bacterial suspension (5.0×10^7) in PBS was added to 100 μ l of serum (LTA antiserum and, as a control, normal rabbit serum) diluted 1/100 in PBS containing 0.1% bovine serum albumin (PBS-BSA). After incubation at 37°C for 40 min, 1.5 ml of PBS-BSA was added to each tube, and the bacteria were deposited by centrifugation. Supernatants were removed, and 100 μ l of 125 I-labeled protein A was added to each tube. The mixture was incubated for another 40 min at 37°C, washed one time in PBS-BSA, and centrifuged. Supernatants were discarded, and the radioactivity of the bacterial pellets was measured in a gamma counter (LKB-Wallac 1260 Multigamma; LKB Sverige AB).

Extraction of LTA from streptococci. Group A streptococcus strain AM23 was grown overnight in 100 ml of Todd-Hewitt broth supplemented with 150 μ Ci of tritiated glycerol (specific activity, 500 mCi/mmol; Radiochemical Centre). The bacteria were harvested and washed five times in distilled water. Extraction was performed on 0.2 g (wet-packed weight) of bacteria suspended in 0.5 ml of distilled water with an equal volume of 90% (wt/vol) phenol at 65°C (42, 44). After being mixed for 5 min, the suspension was centrifuged at $12,000 \times g$ for 30 min. The aqueous phase was removed and dialyzed against six changes of distilled water for 3 days. The presence of LTA in the extract was demonstrated by precipitation with an antiserum against *L. casei* LTA. A 50- μ l amount of LTA antiserum and 25 μ l of tritiated aqueous phenol extract were mixed and diluted with 175 μ l of PBS. Normal rabbit serum was used as a control. After incubation overnight at 37°C, 150 μ l of bovine serum and 800 μ l of 15% PEG 6000 were added to each tube. The mixture was centrifuged at $1,000 \times g$ for 45 min. The pellet was dissolved in scintillation fluid (Biofluor; New England Nuclear Corp.), and the radioactivity was measured in a beta scintillation counter.

RESULTS

Partition of bacteria after enzymatic and heat treatment. The differential effect of proteolytic digestion on bacterial surface structures was utilized to characterize the hydrophobic factor

on the surface of group A streptococci. Four proteolytically modified group A streptococcal strains were partitioned in polymer two-phase systems. Bacteria (5.0×10^8) were suspended in 0.5 ml of enzyme solutions and incubated at 37°C for 30 to 60 min before partition experiments. Trypsin digestion (150 μ g/ml) had a marked effect on the partition of the bacteria (Table 1). Trypsin-treated bacteria lost their hydrophobic character. The addition of increasing amounts of trypsin to group A streptococcus strain AM23 showed that this effect was dose dependent (Fig. 1). Treatment of group A streptococci with pepsin (100 μ g/ml) at pH 4.5 or papain (200 μ g/ml) also resulted in a marked

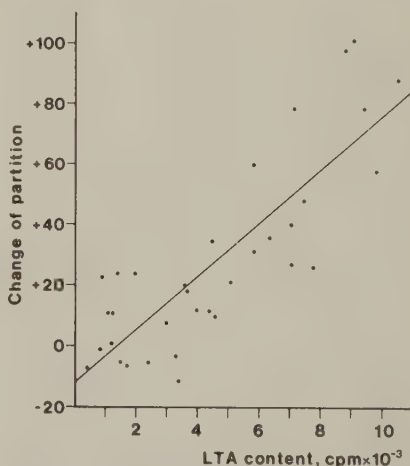


FIG. 4. Correlation between surface hydrophobicity, as measured by hydrophobic affinity partition, and the content of surface LTA. The CP of 34 group A streptococcal strains is plotted against the content of surface LTA expressed as counts per minute ($r = 0.84$). The two-phase systems used are the same as in Fig. 1.

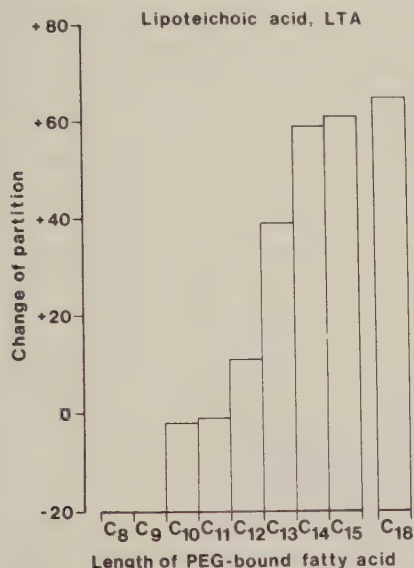


FIG. 5. Effect of PEG-bound fatty acids of varying length on the partition of ^3H -labeled LTA in polymer two-phase systems. For phase composition and calculation of CP see the text.

decrease in CP (Table 1). Digestion with pepsin (100 $\mu\text{g}/\text{ml}$) at the suboptimal pH 5.8 had, however, no significant effect on the partition of the bacteria in polymer two-phase systems (Table 1). Bacterial cells treated with 50 U of hyaluronidase per ml, in most instances, showed a slight increase in CP as compared with the control (Table 1). Heat treatment (100°C for 10 min) did not influence the partitioning of the cells (Table 1).

Effect of PEG-bound fatty acids of various lengths on the partition of group A streptococci. The length of the PEG-bound fatty acid influences the accessibility to the hydrophobic part of the molecules investigated (15, 16). Three group A streptococcal strains were partitioned in phase systems with PEG-bound fatty acids of various lengths. The final concentration of PEG-bound fatty acids in the phase systems was 0.5% (wt/wt). PEG-bound fatty acids C₈ to C₁₂ had a negative effect on the CP (i.e., an increase in the affinity of the bacteria for the dextran-rich bottom phase), whereas C₁₄ (myristic acid) to C₁₈ (stearic acid) fatty acids markedly increased the affinity of the bacteria for the interface and the upper phase (Fig. 2). Similar effects have been observed when serum albumin is partitioned in this type of two-phase system (15).

Determination of surface LTA. Cell wall tei-

choic acids were measured on whole bacterial organisms in a radioimmunoassay utilizing a rabbit antiserum against LTA and ^{125}I -labeled protein A as a second-layer antiantibody. Normal rabbit serum was used as a control. The binding of anti-LTA to group A, C, and G streptococci varied substantially, whereas none of the gram-negative bacteria tested bound any anti-LTA (Fig. 3). Four group A streptococcal strains were treated with proteolytic enzymes before LTA analysis. Trypsin, pepsin (pH 4.5), and papain treatment of the bacteria had a marked effect on the binding of anti-LTA to the bacterial surfaces (Table 2). Pretreatment of bacteria with pepsin at suboptimal pH 5.8 and with hyaluronidase had no significant effect on the binding of anti-LTA to the bacteria (Table 2).

To elucidate the relation between surface LTA and the hydrophobicity of the bacterial cells, 34 group A streptococcal strains were tested in the LTA assay and in hydrophobic affinity partition experiments. The results are presented in Fig. 4 and show a correlation between the binding of anti-LTA and the CP in polymer two-phase systems.

Partition of LTA in polymer two-phase systems. Group A streptococcus strain AM23 was labeled with tritiated glycerol. The LTA of the bacterial preparation was extracted by hot aqueous phenol and tested in polymer two-phase systems containing PEG-bound fatty acids of varying length. As shown in Fig. 5, fatty acids with less than 13 carbon atoms had no significant effect on the partition of LTA, whereas the longer fatty acid chains markedly increased the affinity of the extracted LTA for the upper phase. The high affinity of radiolabeled LTA for St-PEG was retained after treatment of the LTA extract with trypsin (200 $\mu\text{g}/\text{ml}$) or papain (200 $\mu\text{g}/\text{ml}$) for 45 min at 37°C.

Partition of bacteria labeled with different radioisotopes. The influence of different radiolabeling procedures on the hydrophobic affinity partition of bacteria was investigated. Todd-Hewitt broth supplemented with [^3H]thymidine (2 $\mu\text{Ci}/\text{ml}$) was inoculated with a group A streptococcus (strain AM23) and incubated overnight. After washing in PBS, one portion of the bacteria was labeled with ^{51}Cr , another portion was labeled with ^{125}I , and the third portion received no further treatment. After radiolabeling and subsequent washing, the partition of the bacteria was studied in polymer two-phase systems. Bacteria labeled with different radioisotopes behaved similarly when partitioned with various concentrations of St-PEG (Fig. 6). In the phase system without St-PEG, the ^{51}Cr - and ^{125}I -labeled bacteria partitioned more in favor of the top phase as compared with [^3H]thymidine-labeled cells. Control experiments showed that

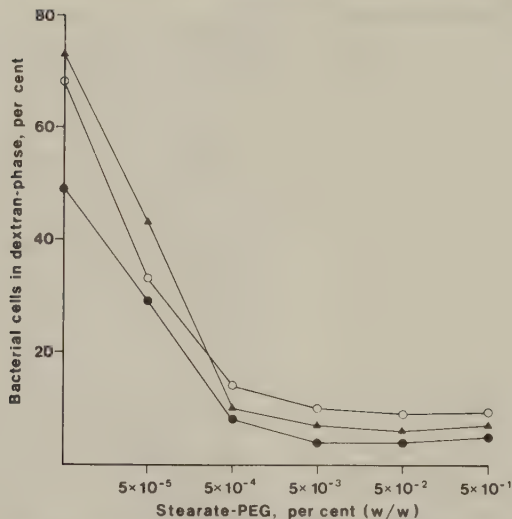


FIG. 6. Effect of different labeling procedures on the partition of a group A streptococcus (strain AM23) in a polymer two-phase system at increasing St-PEG concentrations. The data are presented as the percent bacterial cells in the dextran-rich bottom phase. (For phase composition see the text.) Symbols: bacteria labeled with [³H]thymidine (●), ¹²⁵I (○), and ⁵¹Cr (▲).

this difference was due to the physicochemical conditions of the labeling procedure (i.e., prolonged incubation at 37°C, lactoperoxidase, and hydrogen peroxide) rather than the radioactive isotope itself. All experiments were performed in triplicate. The standard error of the mean never exceeded 6%.

DISCUSSION

It has previously been suggested that M protein of group A streptococci is responsible for the hydrophobicity of these microorganisms (24, 35, 36). M protein has been claimed to mediate the binding of group A streptococci to oral epithelial cells (9). Since hydrophobicity of microorganisms is considered a major determinant for adherence to host cells (20, 28, 32, 38, 39), the importance of M protein therefore seems probable. However, in more recent studies, no significant differences in adherence were found between M-positive and M-negative strains of group A streptococci (3, 8). The removal of M protein by mild pepsin digestion did not abolish the adhering ability of streptococci (8). In the present studies, experiments were performed aimed at defining surface structures responsible for the hydrophobic character of the bacteria.

Bacteria were treated with various proteolytic enzymes with differential effects on surface structures of streptococci. Digestion with trypsin, papain, and pepsin all resulted in a decrease

in hydrophobic affinity of the bacteria (Table 1 and Fig. 1). Mild pepsin digestion at pH 5.8, which is known to cleave the distal portion of the M protein molecule (29), had no significant effect on bacterial surface hydrophobicity (Table 1). These results indicate that the hydrophobic surface structure on group A streptococci either consists of a protein or molecules linked to proteins. Protein solubilized from streptococci by proteolytic digestion often contains LTA (17). Mild pepsin digestion of group A streptococci at a suboptimal pH removes M protein, whereas LTA is retained on the surface (8).

The role of LTA in conferring hydrophobicity was elucidated by assessing the quantity of surface LTAs in a double-layer radioimmunoassay. Antibodies to LTA bound at varying degrees to all group A, C, and G streptococci (Fig. 3). Enzymatic digestion of bacteria before LTA assay revealed that surface LTA was partially removed after treatment with trypsin, papain, and pepsin (Table 2). Mild pepsin digestion had no significant effect on the content of surface LTA (Table 2). This indicates that the surface LTA molecule is bound to a cell wall protein. The nature of the surface protein to which LTA is anchored is unknown. The possibility still remains that LTA binds to a portion of M protein near the surface of the bacteria.

A direct comparison between surface hydrophobicity and LTA was made. The quantity of surface LTA and the degree of surface hydro-

phobicity were determined on a collection of group A streptococci. The results show a correlation between the hydrophobic affinity partition of the bacteria in polymer two-phase systems and the quantity of surface LTA (Fig. 4), indicating that LTA plays a major role in conferring hydrophobicity to group A streptococci. However, the possibility that other surface molecules also contribute to the hydrophobicity of group A streptococci cannot be ruled out.

In further experiments on hydrophobic surface structures, LTA was extracted from intact group A streptococci by the hot aqueous phenol method (42, 44). This extraction procedure yields a preparation of low protein content (44). The LTA extract showed a high affinity for St-PEG when tested in two-phase systems. Treatment of LTA extract with papain, which is known to further lower the protein content of the extract (44), did not influence the hydrophobic affinity of LTA. These results indicate that the hydrophobic affinity expressed by the LTA extract is due to LTA itself rather than a protein bound to LTA.

The length of PEG-bound fatty acids used in the test determines the liability to hydrophobic interactions in polymer two-phase systems (10, 16). Experiments were performed with fatty acids with lengths of 8 to 18 carbon atoms. The partition of whole group A streptococci as well as solubilized LTA required a fatty acid chain of 14 to 13 carbon atoms, respectively, in order to be transferred from the bottom phase into the interface and top phase (Fig. 2 and 5). This similarity in partition behavior between LTA and intact organisms indicates that LTA *in situ* is a major factor responsible for the hydrophobic surface properties of group A streptococci. The difference of one carbon atom indicates that the cell-bound LTA is somewhat restricted in its interaction with PEG-bound fatty acid.

The hyaluronate capsule might mask hydrophobic properties of group A streptococci. Enzymatic removal of hyaluronic acid from bacteria harvested during the stationary phase had no marked effect on either hydrophobic affinity partition or surface LTA (Tables 1 and 2). Hyaluronic acid is known to accumulate on the bacterial surface during logarithmic growth followed by a decrease when the bacteria enter the stationary phase (30). Bacteria were therefore also harvested during the logarithmic growth phase and tested in polymer two-phase systems. Bacteria from these cultures partitioned in a similar way in polymer two-phase systems, indicating that hyaluronic acid has no marked influence upon surface hydrophobicity.

Studies of *Salmonella typhimurium* have revealed that phagocytosis-sensitive R mutants are liable to hydrophobic and ionic interactions,

whereas the smooth phagocytosis-resistant strains have a hydrophilic and uncharged surface (20). The adherence of *E. coli* correlates to their degree of hydrophobicity (28, 32). The importance of hydrophobicity for the phagocytosis of bacteria has been demonstrated by Van Oss (38). Group A streptococci are negatively charged in a physiological milieu (13, 24). Most mammalian cells also carry a net negative charge. LTA on the cell surface of group A streptococci makes these cells markedly hydrophobic. The liability to hydrophobic interactions might serve to overcome the electrical repulsion and thereby facilitate the initial steps in adherence.

A variety of biological properties have been ascribed to LTA. It has been suggested to play a central role in the pathogenesis of streptococcal infections (18, 26, 46). LTA is known to bind to epithelial cells (4, 26), erythrocytes (7, 46), phagocytes (25), and human platelets (5). Hypersensitivity reactions and suppression of the immune response in laboratory animals have been demonstrated to be affected by LTA (14, 23). LTA also acts as a nonspecific T-lymphocyte mitogen (6). The lipid portion of LTA has proved to be essential for biological properties such as immunogenicity (45) and binding of LTA to cell membranes (26). In addition to these properties, the present investigation indicates that LTA is a major hydrophobic constituent of group A streptococci.

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LITERATURE CITED

1. Albertsson, P.-Å. 1971. Partition of cell particles and macromolecules. Wiley-Interscience, New York.
2. Albertsson, P.-Å., and G. D. Baird. 1962. Counter-current distribution of cells. *Exp. Cell Res.* 28:296-322.
3. Alkan, M. L., J. Ofek, and E. H. Beachey. 1977. Adherence of pharyngeal and skin strains of group A streptococci to human skin and oral epithelial cells. *Infect. Immun.* 18:555-557.
4. Beachey, E. H. 1975. Binding of group A streptococci to human oral mucosal cells by lipoteichoic acid. *Trans. Assoc. Am. Physicians* 88:285-292.
5. Beachey, E. H., T. M. Chiang, J. Ofek, and A. H. Kang. 1977. Interaction of lipoteichoic acid of group A streptococci with human platelets. *Infect. Immun.* 16:649-654.
6. Beachey, E. H., J. B. Dale, S. Grebe, A. Ahmed, W. A. Simpson, and J. Ofek. 1979. Lymphocyte binding and T cell mitogenic properties of group A streptococcal lipoteichoic acid. *J. Immunol.* 122:189-195.
7. Beachey, E. H., J. B. Dale, W. A. Simpson, J. D. Evans, K. W. Knox, J. Ofek, and A. J. Wicken. 1979. Erythrocyte binding properties of streptococcal lipoteichoic acids. *Infect. Immun.* 23:618-625.
8. Beachey, E. H., and J. Ofek. 1976. Epithelial cell binding of group A streptococci by lipoteichoic acid on fimbriae denuded of M protein. *J. Exp. Med.* 143:759-771.

9. Ellen, R. P., and R. J. Gibbons. 1972. M-protein-associated adherence of *Streptococcus pyogenes* to epithelial surfaces: prerequisite for virulence. *Infect. Immun.* 5:826-830.
10. Eriksson, E. 1981. Hydrophobic affinity partition of liposomes in aqueous two-phase systems. *J. Chromatogr.* 205:189-193.
11. Eriksson, E., P.-Å. Albertsson, and G. Johansson. 1976. Hydrophobic surface properties of erythrocytes studied by affinity partition in aqueous two-phase systems. *Mol. Cell. Biochem.* 10:123-128.
12. Goding, J. W. 1978. Use of staphylococcal protein A as an immunological reagent. *J. Immunol. Methods* 20:241-253.
13. Hill, M. J., A. M. James, and W. R. Maxted. 1962. Some physical investigations of the behaviour of bacterial surfaces. VIII. Studies on the capsular material of *Streptococcus pyogenes*. *Biochim. Biophys. Acta* 66:264-274.
14. Jackson, D. E., C. R. Howlett, A. J. Wicken, and C. D. F. Jackson. 1981. Induction of hypersensitivity reactions to *Lactobacillus fermentum* and lipoteichoic acid in rabbits. *Int. Arch. Allergy Appl. Immunol.* 65:304-312.
15. Johansson, G. 1976. The effect of poly(ethylene glycol) esters on the partition of proteins and fragmented membranes in aqueous biphasic systems. *Biochim. Biophys. Acta* 451:517-529.
16. Johansson, G., and H. Westrin. 1978. Specific extraction of intact chloroplasts using aqueous biphasic systems. *Plant Sci. Lett.* 13:201-212.
17. Johnson, R. H., and K. L. Vosti. 1977. Purification and characterization of group A streptococcal T-1 antigen. *Infect. Immun.* 16:867-875.
18. Knox, K. W., and A. J. Wicken. 1973. Immunological properties of teichoic acids. *Bacteriol. Rev.* 37:215-257.
19. Magnusson, K.-E., E. Kihlström, L. Norlander, A. Norqvist, J. Davies, and S. Normark. 1979. Effect of colony type and pH on surface charge and hydrophobicity of *Neisseria gonorrhoeae*. *Infect. Immun.* 26:397-401.
20. Magnusson, K.-E., O. Stendahl, C. Tagesson, L. Edebo, and G. Johansson. 1977. The tendency of smooth and rough *Salmonella typhimurium* bacteria and lipopolysaccharide to hydrophobic and ionic interaction as studied in aqueous polymer two-phase systems. *Acta Pathol. Microbiol. Scand. Sect. B* 85:212-218.
21. McCarty, M. 1964. The role of D-alanine in the serological specificity of group A streptococcal glycerol teichoic acid. *Microbiology* 52:259-265.
22. McCahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of protein for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* 29:185-189.
23. Miller, B. A., and R. W. Jackson. 1973. The effect of *Streptococcus pyogenes* teichoic acid on the immune response of mice. *J. Immunol.* 110:148-156.
24. Mörner, H., P.-Å. Albertsson, and G. Kronvall. 1982. Isoelectric points and surface hydrophobicity of gram-positive cocci as determined by cross-partition and hydrophobic affinity partition in aqueous two-phase systems. *Infect. Immun.* 36:227-234.
25. Ofek, J., and E. H. Beachey. 1979. Lipoteichoic-acid-sensitive attachment of group-A streptococci to phagocytes, p. 44-46. *In* M. T. Parker (ed.), *Pathogenic streptococci*. Reedbooks, Ltd., Surrey, England.
26. Ofek, J., E. H. Beachey, W. Jefferson, and G. L. Campbell. 1975. Cell membrane-binding properties of group A streptococcal lipoteichoic acid. *J. Exp. Med.* 141:990-1003.
27. Ofek, J., W. A. Simpson, and E. H. Beachey. 1982. Formation of molecular complexes between a structurally defined M protein and acylated or deacylated lipoteichoic acid of *Streptococcus pyogenes*. *J. Bacteriol.* 149:426-433.
28. Perers, L., L. Andäker, L. Edebo, O. Stendahl, and C. Tagesson. 1977. Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. *Acta Pathol. Microbiol. Scand. Sect. B* 85:308-316.
29. Phillips, G. N., Jr., P. F. Flicker, C. Cohen, B. N. Manjula, and V. A. Fischetti. 1981. Streptococcal M protein: -helical coiled-coil structure and arrangement on the cell surface. *Proc. Natl. Acad. Sci. U.S.A.* 78:4689-4693.
30. Plummer, D. T., A. M. James, H. Gooder, and W. R. Maxted. 1962. Some physical investigations of the behaviour of bacterial surfaces. V. The variation of the surface structure of *Streptococcus pyogenes* during growth. *Biochim. Biophys. Acta* 60:595-603.
31. Smith, H. 1977. Microbial surfaces in relation to pathogenicity. *Bacteriol. Rev.* 41:475-500.
32. Smyth, C. J., P. Jonsson, E. Olsson, O. Söderlind, J. Rosengren, S. Hjertén, and T. Wadström. 1978. Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. *Infect. Immun.* 22:462-472.
33. Stendahl, O., L. Edebo, K.-E. Magnusson, C. Tagesson, and S. Hjertén. 1977. Surface-charge characteristics of smooth and rough *Salmonella typhimurium* bacteria determined by aqueous two-phase partitioning and free zone electrophoresis. *Acta Pathol. Microbiol. Scand. Sect. B* 85:334-340.
34. Stendahl, O., C. Tagesson, and M. Edebo. 1973. Partition of *Salmonella typhimurium* in a two-polymer aqueous phase system in relation to liability to phagocytosis. *Infect. Immun.* 8:36-41.
35. Tylewska, S., S. Hjertén, and T. Wadström. 1979. Contribution of M protein to the hydrophobic surface properties of *Streptococcus pyogenes*. *FEMS Lett.* 6:249-253.
36. Tylewska, S. K., S. Hjertén, and T. Wadström. 1980. Hydrophobic properties of *Streptococcus pyogenes* related to M protein: decrease of surface hydrophobicity and epithelial cell binding by antibiotics. p. 788. *In* J. D. Nelson and C. Grassi (ed.), *Current chemotherapy and infectious disease*, vol 2. American Society for Microbiology, Washington, D.C.
37. Van Driel, D., A. J. Wicken, M. R. Dickson, and K. W. Knox. 1973. Cellular location of the lipoteichoic acids of *Lactobacillus fermenti* NCTC 6991 and *Lactobacillus casei* NCTC 6375. *J. Ultrastruct. Res.* 43:483-497.
38. Van Oss, C. J. 1978. Phagocytosis as a surface phenomenon. *Annu. Rev. Microbiol.* 32:19-39.
39. Wadström, T., C. J. Smyth, A. Faris, P. Jonsson, and J. Freer. 1978. Hydrophobic adsorptive and hemagglutinating properties of enterotoxigenic *Escherichia coli* with different colonization factor antigens and adherence factor, p. 29-45. *In* Neonatal Diarrhoea, VIDO Symposium, October 1978. Stuart Bundle Printing Press, Saskatoon, Canada.
40. Walter, H. 1977. Partition of cells in two-polymer aqueous phases: a surface affinity method for cell separation, p. 307. *In* N. Catsim Poolas (ed.), *Methods of cell separation*. Plenum Publishing Corp., New York.
41. Walter, H., E. J. Krob, and R. Tung. 1976. Hydrophobic affinity partition in aqueous two-phase systems of erythrocytes from different species. *Exp. Cell Res.* 102:14-24.
42. Westphal, O., O. Luderitz, and F. Bister. 1952. Über die Extraktion von Bakterien mit Phenol/Wasser. *Z. Naturforsch.* 7:148-155.
43. Westrin, H., P.-Å. Albertsson, and G. Johansson. 1976. Hydrophobic affinity partition of spinach chloroplasts in aqueous two-phase systems. *Biochim. Biophys. Acta* 436:696-706.
44. Wicken, A. J., J. W. Gibbons, and K. W. Knox. 1973. Comparative studies on the isolation of membrane lipoteichoic acid from *Lactobacillus fermenti*. *J. Bacteriol.* 113:365-372.
45. Wicken, A. J., and K. W. Knox. 1970. Studies on the group F antigen of lactobacilli: isolation of a teichoic acid-lipid complex from *Lactobacillus fermenti* NCTC 6991. *J. Gen. Microbiol.* 60:293-301.
46. Wicken, A. J., and K. W. Knox. 1975. Lipoteichoic acids: a new class of bacterial antigen. *Science* 187:1161-1167.

SURFACE CHARACTERISTICS OF GROUP A STREPTOCOCCI WITH AND WITHOUT M-PROTEIN

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IV

SUMMARY

Twenty M protein-positive and eight M protein-negative strains of group A streptococci were investigated with respect to surface hydrophobicity and amount of lipoteichoic acid (LTA). Surface hydrophobicity as determined in polymer two-phase systems varied substantially between individual strains and there was no correlation to the presence of antiphagocytic M protein. The amount of LTA on the surface of the bacteria varied with the hydrophobic affinity of the cells. Strains with a high content of surface LTA were found among both M-positive and M-negative streptococci. Cellular and extracellular LTA was estimated on six strains by the ability of hot phenol extracts and culture fluids to sensitize erythrocytes and by rocket immunoelectrophoretic quantitation. Differences in content of surface LTA did not correlate to differences in the total amount of cellular LTA. Pepsin digestion of M-positive group A streptococci at suboptimal pH resulted in a loss of M antigen whereas surface LTA and the hydrophobic interaction liability was retained. The results indicate that the degree of surface hydrophobicity as measured by two-phase partitioning is not correlated to neither the typespecific nor the antiphagocytic moiety of M protein. The results rather support the correlation between surface LTA and surface hydrophobicity of group A streptococci.

INTRODUCTION

The importance of hydrophobicity for the adherence of bacteria to mammalian cells has been demonstrated (16, 25, 27, 28, 32, 33). Adherence of group A streptococci to epithelial surfaces has been

suggested to be a prerequisite for the virulence of these organisms (5). It has been claimed that the hydrophobicity of group A streptococci is related to M protein (19, 31, 34) and that M protein mediates the adherence of these organisms to epithelial cells (5). Other studies indicate, however, that there are no differences in adherence between M protein-positive and M protein-negative strains of streptococci (2) and that lipoteichoic acid (LTA) rather than M protein is the adhesin for adherence to epithelial cells (3).

Investigations on Salmonella typhi-murium have shown that the smooth phagocytosis-resistant strains have a hydrophilic and uncharged surface whereas the phagocytosis-sensitive R-mutants are liable to hydrophobic and ionic interactions (16, 29). M protein of group A streptococci is a cell surface antigen responsible for the resistance to phagocytosis by human polymorphonuclear leukocytes (13). The mechanism of the antiphagocytic properties of M protein is unclear (7). It has been suggested that different mechanisms are involved in adherence of group A streptococci to epithelial cells and the recognition of these bacteria by phagocytic cells (3).

Lipoteichoic acid (LTA) is a cell surface component of most gram-positive bacteria (40). We have previously shown that surface associated LTA is the major cell wall component responsible for surface hydrophobicity of group A streptococci (20). Similar evidence for the involvement of glycolipids in the hydrophobic interaction of streptococci with hexadecane droplets have been reported by Ofek et al (24). Surface LTA is released by the action of proteolytic enzymes (3, 20) indicating that LTA is bound to a cell wall protein. Recently, Ofek et al have suggested the formation of molecular complexes between M protein and the polyglycerophosphate backbone of LTA (23).

In the present investigation the surface hydrophobicity and LTA content of M-positive and M-negative strains of streptococci was

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investigated. Surface hydrophobicity of the bacteria was determined by hydrophobic affinity partition (1, 6, 35, 37). Surface LTA and M antigen was determined in double-layer radioimmunoassays (20) and the total amount of cellular and extracellular LTA was estimated by rocket immunoelectrophoresis and red blood cell sensitizing activity. The results indicate that there is no correlation between surface LTA or surface hydrophobicity of the bacteria and their ability to grow in fresh human blood.

MATERIALS AND METHODS

Bacterial Strains. Twenty M protein-positive and eight M protein-negative strains of group A streptococci were obtained from the Collection of Reference Strains of the WHO Collaborating Centre for Reference and Research on Streptococci, Prague, Czechoslovakia. Growth of group A streptococci in human blood was determined essentially as described by Lancefield (12). M-positive strains grew in fresh normal human blood in rotated 3-hours cultures minimally for seven generations. M-negative strains did not grow in fresh human blood obtained from at least two different donors. The M12 strain 255 and the M49 strain W9 were kindly supplied by Dr. Sramek, Prague, Czechoslovakia and by Dr. L.W. Wannamaker, Minneapolis, Minn., respectively. Strains were kept at -70°C in Todd-Hewitt broth supplemented with fetal calf serum. Todd-Hewitt broth was inoculated and incubated for 15-16 h at 37°C . Bacteria were harvested by centrifugation at 3500 rpm for 10 min and washed twice in phosphate buffered saline (PBS; 0.03 M sodium phosphate, 0.12 M NaCl, pH 7.2). The optical density at 620 nm was measured and the bacterial concentration was calculated from a standard curve and adjusted to 1×10^9 organisms per ml.

Radiolabeling of Bacteria. Bacteria were labeled with ^{125}I (sodium iodide; Amersham International Limited, Amersham, England). Fifteen μCi ^{125}I , 5 μl 0.015 % hydrogen peroxide and 5 μl lactoperoxidase (Sigma Chemical Company, Mo. USA) were added to 1×10^9 bacteria in PBS. The mixture was incubated at room temperature for ten minutes. The bacteria were then deposited by centrifugation, washed four times in ice-cold PBS and suspended in PBS to 5×10^8 organisms per ml.

Polymer Two-Phase Systems. Polyethylene glycol (PEG) 4000 (Union Carbide, New York) and dextran T500 (Pharmacia Fine Chemicals, Uppsala, Sweden) were used to prepare polymer two-phase systems (1). Palmitoyl-polyethylene glycol (P-PEG) was kindly supplied by Dr. G. Johansson, University of Lund, Lund, Sweden. The phase systems were prepared from stock solutions of dextran (20%), PEG 4000 (40%), and P-PEG (1%). Salt and buffer stock solutions were made up to concentrations ten to twenty times higher than that of the final concentration in the phase system. Phase systems were prepared and experiments carried out at room temperature. All phase partition experiments were performed in duplicates.

Hydrophobic Affinity Partition Experiments. Single tube partitions were performed with 1.00-g phase systems as described previously (19). The phase systems were composed of 6.1% (wt/wt) dextran, 6.1% (wt/wt) PEG 4000, 0-0.1% (wt/wt) P-PEG, 5.0 mM sodium phosphate buffer (pH 6.9), and 40.0 mM NaCl. Samples were taken from top and bottom phase and determination of the bacteria in the phases was made by measuring the radioactivity in a gamma counter (LKB-Wallac 1270 Rackgamma, LKB Sverige AB, Bromma, Sweden). The change of partition of the bacteria in phase systems with and without P-PEG is presented as described by Magnusson et al (15). Change of partition, CP, was calculated using the following formula:

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$$CP = (T_{P-PEG} - B_{P-PEG}) - (T_{basal} - B_{basal})$$

where T is percent bacteria in polyethylene glycol rich top phase and B is per cent bacteria in dextran rich bottom phase. P-PEG indicates phase system containing polyethylene glycol palmitate and basal indicates phase system without hydrophobic groups. The value of CP can vary in the range 0-200 and is positive when hydrophobic partitioning (i.e. transfer from lower phase to interface or from interface to upper phase) is involved.

Extraction of LTA. Bacteria were grown overnight in 100 ml Todd-Hewitt broth. The cells were deposited by centrifugation and washed five times in distilled water. A pellet containing approximately 5×10^{10} organisms was suspended in 0.6 ml distilled water and heated to 65°C . An equal volume of 90% (wt/vol) hot phenol was added (36, 39). After mixing for 5 min the suspension was centrifuged in the cold at $12,000 \times g$ for 30 min. The aqueous phase was removed and the extraction was repeated once. The combined extracts were dialyzed for 5 days against 6 changes of distilled water.

Anti-LTA antiserum. Rabbit antiserum raised against chloroform-water extracted L. casei 6375 -LTA was a kind gift from Dr. K.W. Knox, Sydney, Australia. The antibody level was 1.63 ng/ml when 0.1 ml was tested in quantitative precipitin reaction against 20 μg LTA extracted by hot phenol from L. casei 6375. This antiserum is specific to the polyglycero-phosphate backbone of LTA (39). All LTA assays (RBC-sensitizing activity, rocket immunoelectrophoresis, and assay of cell-surface LTA) were carried out on the same antiserum from one rabbit.

RBC-Sensitizing Activity of LTA. The sensitizing activity of LTA was assayed essentially as described by Ofek et al (21). Group

O, Rh-negative blood drawn from a healthy donor was heparinized (10 U/ml). Plasma and buffy coat was removed after centrifugation at 500xg for 10 min. The erythrocytes were washed twice in PBS and a 2% (vol/vol) suspension was made in PBS containing 100 μ g of papain and 500 μ g of L-cystein per ml. The mixture was incubated at 37°C for 30 min, washed one time in PBS and resuspended at a concentration of 2% erythrocytes in PBS.

Serial twofold dilutions of LTA (0.1 ml) was made in PBS. Fifty μ l of papain-treated 2% RBC were added to each tube and the mixtures were incubated at 37°C for 45 min. The erythrocytes in each tube were then washed twice in PBS and suspended in 200 μ l of anti-LTA diluted 1/100 in PBS. After incubation at 37°C for 30 min followed by centrifugation at 1.000xg for 5 min, the tubes were gently shaken and agglutination reactions recorded. The reciprocal of the highest dilution which sensitized erythrocytes fully was designated the LTA sensitizing activity.

Estimations of the formation of extracellular lipoteichoic acids were made on cell free neutralized culture fluids after heat-treatment at 56°C for 15 min (17). The culture fluids were concentrated ten times by passing through a Diaflo XM50 filter. The reciprocal of the highest dilution of culture fluid that sensitized erythrocytes completely was designated the culture fluid titer.

Rocket immunoelectrophoresis. Rocket immunoelectrophoresis which will measure both acylated and deacylated LTA was performed on hot phenol extracts after dialysis against distilled water and on culture fluids after dialysis against veronal buffer containing Ca^{++} (14, 38). The electrophoresis was performed in a 0.8% agarose gel in veronal Ca^{++} buffer containing 2% antiserum to L. casei -LTA. The hot phenol extracts were diluted 1/5 whereas the culture fluids were used undiluted. A 10 μ l sample was pipetted into the wells. The gel was pressed, dried and stained with Coomassie

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Brilliant Blue R-250. The amount of LTA in each sample was calculated in relation to a standard. The results were expressed in relation to the mean of all determinations.

Assays of cell-surface LTA. Cell surface teichoic acids were determined on whole bacterial organisms as described previously (19). Fifty μl of bacterial suspension (5×10^8 organisms) was mixed with 100 μl anti-LTA diluted 1/100 in PBS containing 0.1 per cent bovine serum albumin (PBS-BSA). After incubation at 37°C for 40 min the bacteria were washed one time in PBS-BSA and 100 μl ^{125}I -labeled protein A was added to each tube. The mixtures were incubated for another 40 min at 37°C , washed one time in PBS-BSA and centrifuged. Supernatants were discarded and the radioactivity of the bacterial pellets was measured in a gamma counter.

Assay of M antigen. Type-specific M antigens on whole group A streptococci were determined in a radioimmunological assay as described elsewhere (Mörner, H. and K. Nivenius Larsson. Submitted for publication). Hyperimmune anti-M12 and M49 antisera were prepared in rabbits by i.v. immunization with whole streptococci. Antisera were diluted 1/5 in PBS and adsorbed with the group A streptococcal strain M4/118. Normal rabbit serum used as a control was also absorbed in the same manner. One hundred μl of bacterial suspension (1×10^8 organisms) was mixed with 100 μl absorbed anti M antiserum diluted 1/100 in PBS-BSA. The mixture was incubated, washed and treated with ^{125}I -labeled protein A as described above for assay of cell-wall LTA. The absorbed antisera were tested against 10 homologous serotypes, 48 heterologous serotypes and 8 M negative strains. All homologous serotypes gave a unequivocal reaction distinct from the weaker reaction with the heterologous serotypes. The reaction with homologous serotypes was highly significant ($p < 0.001$)

Enzymatic digestion of group A streptococci. Four heat-stabilized (80°C 5 min) group A streptococcal strains were treated with increasing amounts of pepsin at suboptimal pH. Bacteria were washed once in 0.1 M phosphate buffer, pH 5.8, and 1.0×10^9 organisms were suspended in the same buffer containing varying amounts of pepsin. The mixture was incubated at 37°C for 45 min and the reaction was terminated by the addition of 100 μl of 1M Tris base. Bacteria were then washed twice in ice-cold PBS and resuspended in appropriate solutions for use in polymer two-phase system, LTA-assay and M antigen assay.

RESULTS

Hydrophobic Affinity Partition. Relative surface hydrophobicity of 20 M protein-positive and 8 M protein-negative group A streptococcal strains was determined by hydrophobic affinity partition. The investigated bacteria varied greatly in affinity for PEG-palmitate (Table I). Bacteria with high hydrophobic affinity were found among M protein-positive as well as M protein-negative strains.

Determination of surface LTA. Surface associated lipoteichoic acids were determined on whole bacterial organisms in a radioimmunoassay utilizing a rabbit antiserum against LTA and ^{125}I -labeled protein A as a second layer anti-antibody. Normal rabbit serum was used as a control. The binding of anti-LTA varied substantially between individual strains (Table I). Bacteria with a high content of surface LTA were found among both M protein-positive and M protein-negative strains. The obtained data were submitted to statistical evaluation using the Wilcoxon test (18). The differences in surface LTA content between M-positive and M-negative strains was highly significant. Differences in surface hydrophobicity (change of partition) between M-positive and M-negative strains was, however, not significant when submitted to the same statistical evaluation.

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Table I. Hydrophobic affinity partition and content of surface LTA of group A streptococci

Bacterial strains	M-protein	Surface hydrophobicity index Change of partition	LTA content, cpm $\times 10^{-3}$
M 1/103	+	- 1.9	6.0
M 4/118	+	50.6	10.5
M 8/104	+	8.7	4.0
M12/103	+	25.5	2.6
M13/119	+	71.3	9.0
M22/120	+	12.7	4.9
M23/108	+	8.0	2.0
M24/109	+	- 0.6	2.3
M25/174	+	43.4	7.8
M28/169	+	22.2	7.1
M36/114	+	21.5	4.7
M37/196	+	17.2	6.0
M38/169	+	19.4	6.8
M46/105	+	4.2	5.0
M49/123	+	58.6	11.4
M53/134	+	61.3	9.6
M54/193	+	- 2.4	6.5
M56/123	+	34.1	9.2
M58/126	+	8.0	7.2
M66/131	+	44.8	8.1
T 1	-	11.0	5.2
T 2	-	8.7	4.2
T11	-	8.4	1.9
T12	-	21.6	3.0
T13	-	75.0	8.6
T15	-	0.6	2.5
T23	-	9.8	1.5
T26	-	11.5	1.5

Inter-assay variation. Surface hydrophobicity and surface LTA of four strains of heat inactivated bacteria (80°C 5 min) was determined on five different occasions (fig. 1). Strains (M24, M49, M53 and T1) with varying degrees of surface hydrophobicity were selected. The same batch of phase system was used throughout the test. Linear regression analysis showed a correlation between the two parameters (change of partition and LTA content) within the range measured ($r = 0.86$). As seen in fig. 1 the inter-assay variation was larger for change of partition in polymer two-phase system then for LTA content.

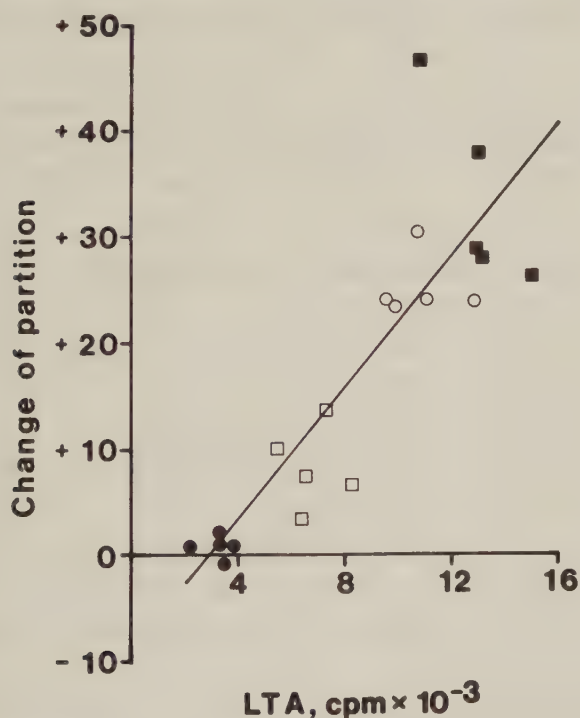


Figure 1. Surface hydrophobicity (expressed as change of partition) and surface LTA of four strains of group A streptococci determined on five different occasions. Bacterial strains ●, M24; ○, M49; ■, M53; □, T1. Correlation coefficient = 0.86.

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Intra-assay variations. Two group A streptococcal strains (M53 and T1) were assayed 10 times for surface hydrophobicity and surface LTA. The intra-assay variation (coefficient of variation in per cent) for change of partition in polymer two-phase system was 4.6% for strain M53 and 15.5% for strain T1. The intra-assay variation for LTA content was 8.0% and 7.4%, respectively.

Table II. Comparison of relative amounts of surface LTA, total cellular and extracellular LTA produced by six group A streptococcal strains

Bacterial strain	M protein	Surface LTA cpm $\times 10^{-3}$	Cellular LTA		Extracellular LTA	
			LTA- titer	"Rocket" titer	Culture- fluid titer	"Rocket" titer
M23/108	+	1.4	40-80	0.8	80	1.2
M24/109	+	0.6	80	0.9	80	0.8
M49/123	+	6.9	80	1.5	80	0.8
M53/134	+	5.8	40-80	0.8	80	1.1
T13	-	4.0	40-80	1.5	40	1.2
T15	-	2.9	40	0.4	40	0.8

- a) Reciprocal of the highest dilution of hot phenol extracts that fully sensitized erythrocytes
- b) Relative amounts of cellular and extracellular LTA estimated by rocket immunoelectrophoresis. See the text
- c) Reciprocal of the highest dilution of culture fluid that fully sensitized erythrocytes

Production of LTA. Suspensions of six group A streptococcal strains were extracted with hot aqueous phenol for comparison of the relative amounts of LTA in the organisms. A semiquantitative estimation of acylated LTA was obtained by comparing the extent to which serial two-fold dilutions of the extracts and culture fluids retained the red blood cell sensitizing activity. Rocket immunoelectrophoresis which detects both acylated and deacylated LTA was also performed. As seen in Table II the concentration of acylated LTA in the extracts and culture fluids from individual strains did not vary significantly although the quantity of surface LTA measured on the bacteria prior to phenol extraction varied markedly. The amounts of LTA measured by rocket immunoelectrophoresis (acylated and deacylated LTA) differed between individual strains but did not correlate to the differences in the amounts of surface associated LTA (Table II).

Effect of pepsin digestion on surface LTA, M antigen and partition of bacteria. Two group A streptococcal strains (M type 12 and 49) were pretreated with pepsin at suboptimal pH and tested in hydrophobic affinity partition, LTA assay and M antigen assay. Pepsin digestion at suboptimal pH has been claimed to remove M protein whereas LTA is retained on the surface (3). As seen in table III already mild pepsin digestion (10 $\mu\text{g/ml}$) has an effect on the amount of M antigen. In contrast, pretreatment of bacteria with pepsin had no marked effect on the binding of anti-LTA to their surfaces or change of partition in polymer two-phase systems (Table III).

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Table III. Effects of pepsin treatment (pH 5.8) of group A streptococci on the partition of the bacteria in polymer two-phase systems and the binding of antibodies to LTA and M antigen to their surfaces

Bacterial strain	Pepsin $\mu\text{g/ml}$	Antibody binding($\text{cpm} \times 10^{-3}$)		Index surface hydrophobicity change of partition
		anti-LTA	anti-M	
255	-	4.2	46.7	24.5
	10	4.8	32.2	22.6
	100	4.9	20.2	24.4
	1000	4.8	9.9	18.4
W9	-	8.4	23.9	29.2
	10	9.3	20.1	34.5
	100	9.5	13.9	31.3
	1000	9.7	12.1	37.2

Partition of Bacteria after Pretreatment with Human Serum. Eight bacterial strains were preincubated with human serum before hydrophobic affinity partition. A 100 μl portion of normal human serum, diluted 1:10 in PBS, was added to 1×10^9 bacteria and incubated at room temperature for 30 min. The bacteria were then washed twice in PBS and suspended in sterile water. The surface hydrophobicity of the bacteria pretreated with serum was determined in polymer two-phase systems and compared with the same bacteria pretreated with PBS alone as a control. The hydrophobic affinity was in all instances higher after pretreatment with human serum (Fig. 2). The effect was more pronounced with bacteria having a hydrophobic surface prior to pretreatment with human serum.

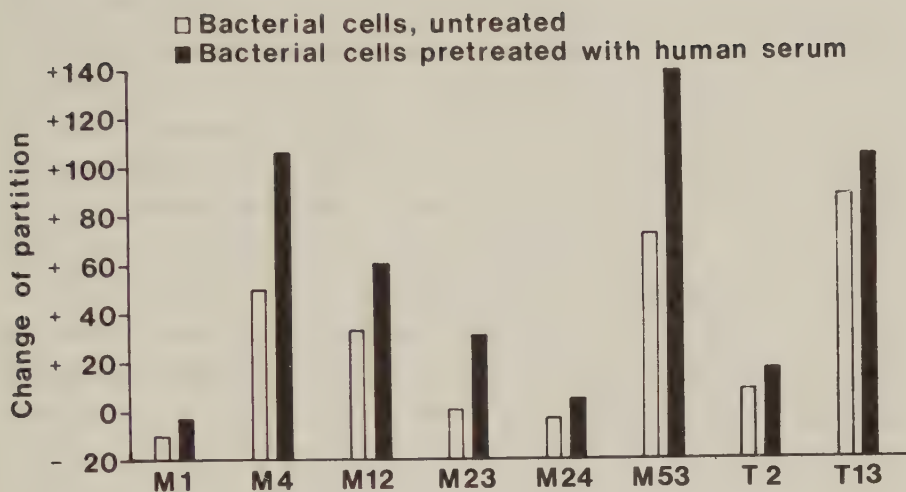


Figure 2. Effect of normal human serum on hydrophobic affinity partition of six M-positive and two M-negative group A streptococcal strains.

DISCUSSION

We have previously suggested that surface associated LTA rather than M protein determines the degree of surface hydrophobicity of group A streptococci (20). The present investigation was undertaken to describe the surface properties and quantity of surface LTA of group A streptococci in relation to their ability to grow in fresh human blood. The quantity of surface associated LTA on streptococci was assessed in a double-layer radioimmunoassay (20). Statistical evaluation of the data on surface LTA content indicate significant differences between M-positive and M-negative strains. However, looking at individual strains it is evident that bacteria

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with a high hydrophobic affinity and a high content of surface LTA are found among both M-positive and M-negative strains (Table I). In addition several M-positive strains showed a low hydrophobic interaction liability and a low surface LTA content (Table I). Our results suggest that the LTA content and surface hydrophobicity of group A streptococci does not correlate to the presence of M protein. The strains used in this study were selected by a functional rather than biochemical definition of the M antigen. The development of a new M antigen assay (Miörner, H. and K. Nivenius Larsson. Submitted for publication) enabled us to measure M antigen on the surface of whole streptococci. It has been suggested that mild pepsin treatment cleaves the distal portion of M protein (26). M antigen, surface LTA and surface hydrophobicity was determined on two group A streptococcal strains before and after treatment with varying concentrations of pepsin at suboptimal pH 5.8. Pepsin treatment resulted in a loss of M antigen whereas the amount of surface LTA and the degree of surface hydrophobicity was unaffected or slightly increased. Taken together this would indicate that neither the antiphagocytic nor the antigenic determinants of M protein contribute to the hydrophobic properties of group A streptococci. Furthermore, one can conclude that surface LTA is not bound to the distal part of M protein. The possibility still remains, however, that LTA is bound to a proximal part of the M protein which does not exert any antiphagocytic or M type antigen properties.

We have previously described marked changes of surface properties of gram-positive bacteria after binding of human serum proteins (21). Pretreatment of streptococci with human serum leads to an increase in isoelectric point of the bacteria (19). In the present study M-positive and M-negative strains of streptococci were pretreated with human serum prior to hydrophobic affinity partition. All strains tested showed an increase in hydrophobic affinity after serum treatment (Fig. 2). The change of partition

was larger in strains that had a hydrophobic surface prior to serum treatment. The nonlinear relationship between change of partition and hydrophobicity indicated in fig. 1 could account for the limited increase in change of partition seen with the strains of low hydrophobic affinity. The biological significance of increased hydrophobicity and more neutral isoelectric point after serum treatment of streptococci is not clear but has to be taken into account in studies of virulence.

In the present study the bacteria were grown in Todd-Hewitt broth for 15-16 hours. It has been reported that physicochemical surface properties and LTA production can vary in response to differences in the environment (8, 9, 17, 38). This possible effect of different growth media and growth conditions on the surface properties of the M-positive and M-negative strains has not been studied systematically.

An increasing number of receptors for human proteins in streptococcal species has recently been discovered (11). It has been suggested that aggregated β_2 -microglobulin binds to M-protein (4). The binding of β_2 -microglobulin to M-positive and M-negative strains of streptococci does not correlate to the surface LTA content or hydrophobicity of the strains (L. Björck, H. Miörner, O. Kühnemund, G. Kronvall, R. Sundler, unpublished). It has also been suggested that M protein carries a receptor for human fibrinogen (10). No correlation could be found, however, between the binding of fibrinogen to the various M-positive and M-negative strains and the content of surface LTA (unpublished observation). These data indicate that LTA is not bound to the part of the M protein molecule involved in the binding of β_2 -microglobulin and fibrinogen.

The reproducibility of the hydrophobic affinity partition and LTA assay was investigated on four group A streptococcal strains. The same batch of heat inactivated bacteria were tested on five different occasions (Fig. 1). The intra-assay variation was also determined on two group A streptococcal strains. The results indi-

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cate an acceptable discriminating power of both tests. If fresh bacteria are used, however, there is a more pronounced biological variation which emphasizes the need to test both parameters (LTA and change of partition) on the same bacterial preparation.

Large differences in the quantity of surface LTA were seen between individual strains of M-positive and M-negative streptococci (Table I). These differences could reflect a variation in the total amount of cellular LTA or a variation in the amount of surface protein to which LTA is bound. Hot phenol extracts of LTA from six streptococcal strains showed no significant difference in the total amount of acylated LTA as estimated by erythrocyte sensitizing activity (Table II). Rocket immunoelectrophoresis which measures both acylated and deacylated LTA revealed differences between individual strains but these variations did not correlate to the content of surface LTA (Table II). The results in Table II would indicate that the total amount of extracellular LTA is equal to the total amount of LTA extracted from the cells. We have not, however, determined the efficacy of the hot phenol extraction procedure and, consequently, this comparison can not be made.

We conclude that surface LTA rather than M protein is responsible for the hydrophobic character of group A streptococci and that there is no correlation between the surface hydrophobicity of group A streptococci as measured by two-phase partitioning and their proneness to phagocytosis by human polymorphonuclear leukocytes. The possibility that different mechanisms are involved in adherence of streptococci to epithelial cells and to phagocytes has previously been suggested (3, 30). Our study further emphasizes the importance of distinguishing between different virulence promoting properties of group A streptococci.

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REFERENCES

1. Albertsson, P.-Å.: Partition of cell particles and macromolecules. Wiley-Interscience, New York, 1971.
2. Alkan, M.L., Ofek, J. and Beachey, E.H.: Adherence of pharyngeal and skin strains of group A streptococci to human skin and oral epithelial cells. *Infect. Immun.* 18: 555-557, 1977.
3. Beachey, E.H. and Ofek, J.: Epithelial cell binding of group A streptococci by lipoteichoic acid on fimbriae denuded of M protein. *J. Exp. Med.* 143: 759-771, 1976.
4. Björk, L., Tylewska, S.K., Wadström, T. and Kronvall, G.: β_2 -microglobulin is bound to streptococcal M protein. *Scand. J. Immunol.* 13: 391-394, 1981.
5. Ellen, R.P. and Gibbons, R.J.: M-protein-associated adherence of Streptococcus pyogenes to epithelial surfaces: prerequisite for virulence. *Infect. Immun.* 5: 826-830, 1972.
6. Eriksson, E., Albertsson, P.-Å. and Johansson, G.: Hydrophobic properties of erythrocytes studied by affinity partition in aqueous two-phase systems. *Mol. Cell. Biochem.* 10: 123-128, 1976.
7. Fox, E.N.: M proteins of group A streptococci. *Bact. Rev.* 38: 57-86, 1974.
8. Hardy, L., Jacques, A., Forester, H., Campbell, L.K., Knox, K.W. and Wicken, A.J.: Effect of fructose and other carbohydrates on the surface properties, lipoteichoic acid production and extracellular proteins of Streptococcus mu-

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- tans Ingbritt grown in continuous culture. Infect. Immun. 31: 78-87, 1981.
9. Jacques, N.A., Hardy, L., Knox, K.W. and Wicken, A.J.: Effect of growth conditions on the formation of extracellular lipoteichoic acid by Streptococcus mutans BHT. Infect. Immun. 25: 75-84, 1979.
10. Kantor, F.S.: Fibrinogen precipitation by streptococcal M protein. I. Identity of the reactants, and stoichiometry of the reaction. J. Exp. Med. 121: 849-859, 1965.
11. Kronvall, G., Björck, L., Miörner, H., Myhre, E. and Widebäck, K.: Receptors for mammalian proteins in streptococcal species of human and animal origin. p 219-220. In S.E. Holm and P. Christensen (eds), Basic Concepts of Streptococci and Streptococcal Diseases. Reedbooks Ltd., Chertsey, 1982.
12. Lancefield, R.C.: Differentiation of group A streptococci with a common R-antigen into three serological types, with special reference to the bactericidal test. J. Exp. Med. 106: 525-544, 1957.
13. Lancefield, R.C.: Current knowledge of type-specific M antigens of group A streptococci. J. Immunol. 89: 307-313, 1962.
14. Laurell, C.-B.: Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal. Biochem. 15: 45-52, 1966.
15. Magnusson, K.-E., Kihlström, E., Norlander, L., Norqvist, A., Davies, J. and Normark, S.: Effect of colony type and pH on surface charge and hydrophobicity of Neisseria gonorrhoeae. Infect. Immun. 26: 379-401, 1979.
16. Magnusson, K.-E., Stendahl, O., Iagesson, C., Edebo, L. and Johansson, G.: The tendency of smooth and rough Salmonella typhimurium bacteria and lipopolysaccharide to hydrophobic and ionic interaction as studied in aqueous polymer two-phase systems. Acta pathol. microbiol. scand. Sect. B. 85: 212-218, 1977.
17. Markham, J.L., Know, K.W., Wicken, A.J. and Hewett, M.J.: Formation of extracellular lipoteichoic acid by oral streptococci and lactobacilli. Infect. Immun. 12: 378-386, 1975.
18. Milton, R.C.: An extended table of critical values for the Mann-Whitney (Wilcoxon) two sample statistic. J. Amer. Statist. Ass. 59: 925-934, 1964.
19. Miörner, H., Albertsson, P.-Å. and Kronvall, G.: Isoele-

- tric points and surface hydrophobicity of gram-positive cocci as determined by cross-partition and hydrophobic affinity partition in aqueous two-phase systems. Infect. Immun. 36: 227-234, 1982.
20. Miörner, H., Johansson, G. and Kronvall, G.: Lipoteichoic acid is the major cell wall component responsible for surface hydrophobicity of group A streptococci. Infect. Immun. 39: 336-343, 1983.
 21. Miörner, H., Myhre, E., Björck, L. and Kronvall, G.: Effect of specific binding of human albumin, fibrinogen, and immunoglobulin G on surface characteristics of bacterial strains as revealed by partition experiments in polymer phase systems. Infect. Immun. 29: 879-885, 1980.
 22. Ofek, J., Beachey, E.H., Jeffersson, W. and Campbell, G.L.: Cell membrane-binding properties of group A streptococcal lipoteichoic acid. J. Exp. Med. 141: 990-1003, 1975.
 23. Ofek, J., Simpson, W.A. and Beachey, E.H.: Formation of molecular complexes between a structurally defined M protein and acylated or deacylated lipoteichoic acid of Streptococcus pyogenes. J. Bacteriol. 149: 426-433, 1982.
 24. Ofek, J., Whitnack, E. and Beachey, E.H.: Hydrophobic interactions of group A streptococci with hexadecane droplets. J. Bacteriol. 154: 139-145, 1983.
 25. Perers, L., Andåker, L., Edebo, L., Stendahl, O. and Tagesson, C.: Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. Acta pathol. microbiol. scand. Sect. B. 85: 308-316, 1977.
 26. Phillips, G.N., Jr., Flicker, P.F., Cohen, C., Manjula, B.N. and Fischetti, V.A.: Streptococcal M protein: α -Helical coiled-coil structure and arrangement on the cell surface. Proc. Natl. Acad. Sci. U.S.A. 78: 4689-4693, 1981.
 27. Smyth, C.J., Jonsson, P., Olsson, E., Söderlind, O., Rosengren, J., Hjertén, S. and Wadström, I.: Differences in hydrophobic surface characteristics of porcine enteropathogenic Escherichia coli with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect. Immun. 22: 462-472, 1978.
 28. Stendahl, O., Edebo, L., Magnusson, K.-E., Tagesson, C. and Hjertén, S.: Surface-charge characteristics of smooth and

rough Salmonella typhimurium bacteria determined by aqueous two-phase partitioning and free zone electrophoresis. Acta pathol. microbiol. scand. Sect. B. 85: 334-340, 1977.

29. Stendahl, O., Tagesson, C. and Edebo, L.: Partition of Salmonella typhimurium in a two-polymer aqueous phase system in relation to liability to phagocytosis. Infect. Immun. 8: 36-41, 1973.
30. Swanson, E., Sparks, J.E., Young, D. and King, G.: Studies on gonococcus infection. X. Pili and leucocytes association factor as mediators of interactions between gonococci and eukaryotic cells in vitro. Infect. Immun. 11: 1352-1361, 1975.
31. Tylewska, S., Hjertén, S. and Wadström, I.: Contribution of M protein to the hydrophobic surface properties of Streptococcus pyogenes. FEMS Microbiol. Lett. 6: 249-253, 1979.
32. Van Oss, C.J.: Phagocytosis as a surface phenomenon. Ann. Rev. Microbiol. 32: 19-39, 1978.
33. Wadström, I., Smyth, C.J., Faris, A., Jonsson, P. and Freer, J.: Hydrophobic adsorptive and hemagglutinating properties of enterotoxigenic Escherichia coli with different colonization factor antigens and adherence factor. p. 29-45. In "Neonatal Diarrhoea" VIDO Symposium, Stuart Bundle Printing Press, Saskatoon, Canada, 1978.
34. Wadström, I. and Tylewska, S.: Surface hydrophobicity of group A streptococci. J. Infect. Dis. 146: 576, 1982.
35. Walter, H., Krob, E.J. and Tung, R.: Hydrophobic affinity partition in aqueous two-phase systems of erythrocytes from different species. Exp. Cell. Res. 102: 14-24, 1976.
36. Westphal, O., Luderitz, O. and Bister, F.: Über die extraktion von bakterien mit phenol/wasser. Z. Naturforsch. 7b: 148-155, 1952.
37. Westrin, H., Albertsson, P.-Å. and Johansson, G.: Hydrophobic affinity partition of spinach chloroplasts in aqueous two-phase systems. Biochim. Biophys. Acta 436: 696-706, 1976.
38. Wicken, A.J., Broady, K., Ayres, A. and Knox, K.W.: Production of lipoteichoic acid by lactobacilli and Streptococci grown in different environments. Infect. Immun. 36: 864-869, 1982.
39. Wicken, A.J., Gibbens, J.W. and Knox, K.W.: Comparative studies on the isolation of membrane lipoteichoic acid from Lac-

tobacillus fermenti. J. Bact. 113: 365-372, 1973.

40. Wicken, A.J. and Knox, K.W.: Lipoteichoic acids: A new class of bacterial antigen. Science 187: 1161-1167, 1975.

ASSAY OF TYPE-SPECIFIC M ANTIGENS ON WHOLE GROUP A STREPTOCOCCI

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ABSTRACT

A novel radioimmunoassay of type specific M antigens on whole group A streptococcal cells is described. Absorbed rabbit anti-M antisera directed against M types 12 and 49 were used for determining M antigens on intact bacterial organisms. Staphylococcal protein A labelled with ^{125}I was used as an anti-antibody reagent. The absorbed antisera were tested against 10 homologous and 48 heterologous serotypes. All homologous serotypes gave a unequivocal reaction distinct from the weaker reaction with the heterologous serotypes. The type-specificity of the reaction was confirmed by the removal of type-specific antibodies after absorption to purified M protein coupled to Sepharose 4B. The results indicate that the described method is a simple and reliable technique for the recognition of M types of group A streptococci and offers a valuable tool for studies of M antigen in situ.

INTRODUCTION

Streptococcal M proteins are antiphagocytic surface antigens responsible for the virulence of group A streptococci. More than 60 different M antigens have been discovered. The recognition of these M antigens has special significance for epidemiological studies. Several methods have been described for the assay of M antigens and antibodies (1). Commonly, the recognition of different M types of group A streptococci has been accomplished by the capillary precipitin test (2). The precipitin reaction is performed with precipitating antisera absorbed with organisms of heterologous M types. In other methods such as the Ouchterlony immunodiffusion assay described by Michael and Massell (3) un-

absorbed antisera were used. An assay of streptococcal M protein on whole cells using an immunofluorescence method has been described by Fox (4). The present paper describes a radioimmunoassay of type-specific M antigens on whole streptococci utilizing absorbed rabbit anti-M antisera and ^{125}I labelled protein A as a second layer anti-antibody.

MATERIALS AND METHODS

Bacterial Strains. Group A streptococci (M types 12 and 49) originating from different outbreaks of group A streptococcal infections were kindly provided by the late Dr. L.W. Wannamaker, Minneapolis, Minn. A collection of representative strains of Streptococcus pyogenes for established M types were from the Collection of Reference Strains of the WHO Collaborating Centre for Reference and Research on Streptococci, Prague, Czechoslovakia. This collection also included 8 M-negative strains defined as lacking M antigen in serum bactericidal test. Strains were stored at -70°C in Todd-Hewitt broth (Difco Laboratories, Detroit, MI.) supplemented with fetal calf serum. Todd-Hewitt broth cultures were incubated overnight at 37°C . Bacteria were harvested by centrifugation at 3000 rpm for 15 min and washed twice in phosphate-buffered saline (PBS) (0.03 M sodium phosphate, 0.12 M NaCl, pH 7.2). The optical density at 620 nm was measured and the bacterial concentration was calculated from a standard curve and adjusted to 10^9 organisms per ml. The bacteria were then heat stabilized at 80°C for 5 min and suspended in PBS containing 0.02 % sodium azide.

Antisera. Rabbit antiserum against M type 12 and M type 49 was kindly supplied by Dr. J. Havlicek, Prague, Czechoslovakia. The rabbit antisera were prepared by intravenous immunization with

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whole streptococci.

Absorption of Antisera . The reference group A streptococcal strain M4/118 was used for absorption of antisera. A young culture was inoculated into 500 ml of Todd-Hewitt broth and incubated for 40 h at 37°C. Bacteria were killed by heating for 30 min at 60°C and harvested by centrifugation. The cells were washed three times in PBS and stored in PBS containing 0.02% sodium azide until used. Absorption was performed by mixing the bacterial pellet with 0.5 ml antiserum diluted 1/5 in PBS. After 1 h at room temperature, the bacteria were deposited by centrifugation and the antiserum was collected. The normal rabbit serum used as a control was also absorbed in the same manner.

Affinity chromatography. Highly purified M protein (5) was kindly provided by Dr. O. Kühnemund, Jena, GDR. The M12 protein was CNBr-coupled (6) to Sepharose 4B (1 mg protein/ml gel). Glycine-Sepharose 4B was used as control. Pasteur pipetes were plugged with glas wool and packed with 0.5 ml gel. The gels were washed with 5 ml PBS and then 300 µl anti-M 12 antiserum diluted 1/15 in PBS was allowed to drain into the gels. The anti-M 12 antiserum had previously been absorbed with the group A streptococcal strain M4/118 as described above. The columns were incubated at room temperature for 2 h and then washed with 1.8 ml PBS. Antibodies absorbed to the columns were eluted with 0.1 M glycine buffer, pH 2.0 and dialysed for 2 days against 3 changes of PBS.

M Antigen Assay. Absorbed anti-M antisera were used for determining M antigens on whole streptococcal cells. Normal rabbit serum was used as a control. Streptococcal protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) was labelled with ¹²⁵I by the chloramine-T method (7) and used as a second layer anti-antibody. The M antigen assay was performed in duplicate in plastic tubes

(70 by 12 mm; AB Cerbo, Trollhättan, Sweden) by adding 1×10^8 bacteria ($100 \mu\text{l}$ suspension containing 10^9 organisms per ml in PBS) to $100 \mu\text{l}$ of antiserum diluted 1/100 in PBS containing 0.1% bovine serum albumin (PBS-BSA). After incubation at 37°C for 40 min 1.5 ml of PBS-BSA was added to each tube and the bacteria were deposited by centrifugation. Supernatant fluids were removed and $100 \mu\text{l}$ ^{125}I labelled protein A was added to each tube. The mixture was incubated for another 40 min at 37°C followed by one wash in PBS-BSA. After centrifugation, the supernatant fluids were removed and the radioactivity of the bacterial pellets in the test tubes was measured in a gamma counter (LKB Rack Gamma, model 1270; LKB Sverige AB, Bromma, Sweden).

RESULTS

Absorption of Antisera

Rabbit antisera were diluted 1/5 in PBS and absorbed with the streptococcal reference strain M4/118. The binding of unabsorbed and absorbed antisera to three heterologous and three homologous group A streptococcal strains was determined in a radioimmunoassay utilizing 125 I-labelled protein A as second layer anti-antibody (Figure 1). Both antisera were used in a final dilution of 1/100 in PBS. As seen in Figure 1 this absorption procedure removes most of the cross-reacting antibodies without any substantial loss of type-specific antibodies.

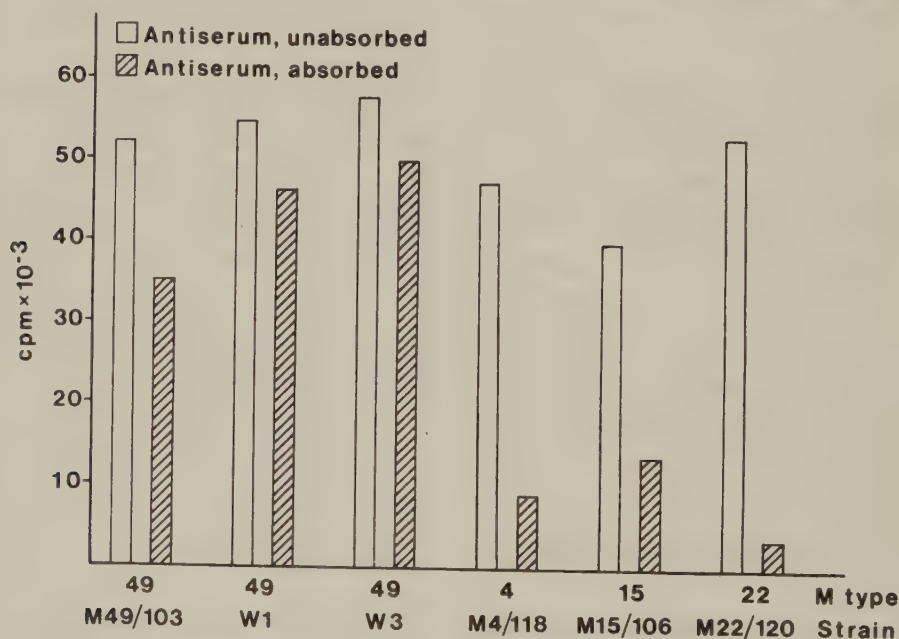


Figure 1: Reaction of unabsorbed and absorbed anti M49 antiserum with 3 homologous and 3 heterologous strains. The M type 4 strain was used for absorption.

Specificity of the M Reaction

The binding of absorbed anti M12 and anti M49 antisera to the surface of 48 heterologous serotypes, ten homologous serotypes and eight M negative strains was determined in a radioimmuno assay utilizing ^{125}I -labelled protein A as a second layer anti-antibody (Figures 2 and 3). The mean cpm values $\times 10^{-3}$ for the homologous reaction was 26.6 (SD=3.7, range 21.7-32.0) for M type 12 and 54.3 (SD=7.9, range 37.4-59.8) for M type 49. Mean values for all heterologous serotypes was 8.0 (SD = 2.4, range 3.5-13.2) for anti-M 12 and 11.5 (SD = 6.8, range 3.0-25.1) for anti-M 49. The reaction with homologous serotypes was highly significant ($p < 0.001$). Heat treatment of the bacteria (80°C 5 min) did not alter their antibody binding capacity. Mean cpm value $\times 10^{-3}$ for controls consisting of normal rabbit serum was 2.4 (SD = 0.7, range 1.5-6.6).

The specificity of the M 12 reaction was also tested by absorption of anti-M 12 antiserum with purified M 12 protein coupled to Sepharose 4B. Glycine-Sepharose 4B was used as control. The antiserum absorbed with M 12-Sepharose and Glycine-Sepharose was tested in the described M antigen assay against a M type 12 strain and against a heterologous M type 15 strain (Table 1). The absorption of antiserum with M 12-Sepharose resulted in a marked decrease in reactivity when tested against the homologous M 12 strain as compared to the same antiserum absorbed with Glycine-Sepharose. No such loss in reactivity was observed when the same absorbed antisera were tested against a heterologous M type 15 strain. Elution of bound antibodies was carried out using 0.1 M glycine buffer, pH 2. The material eluted from M 12-Sepharose showed a low but significant binding to the M type 12 strain whereas no reactivity was seen in the material eluted from Glycine-Sepharose.

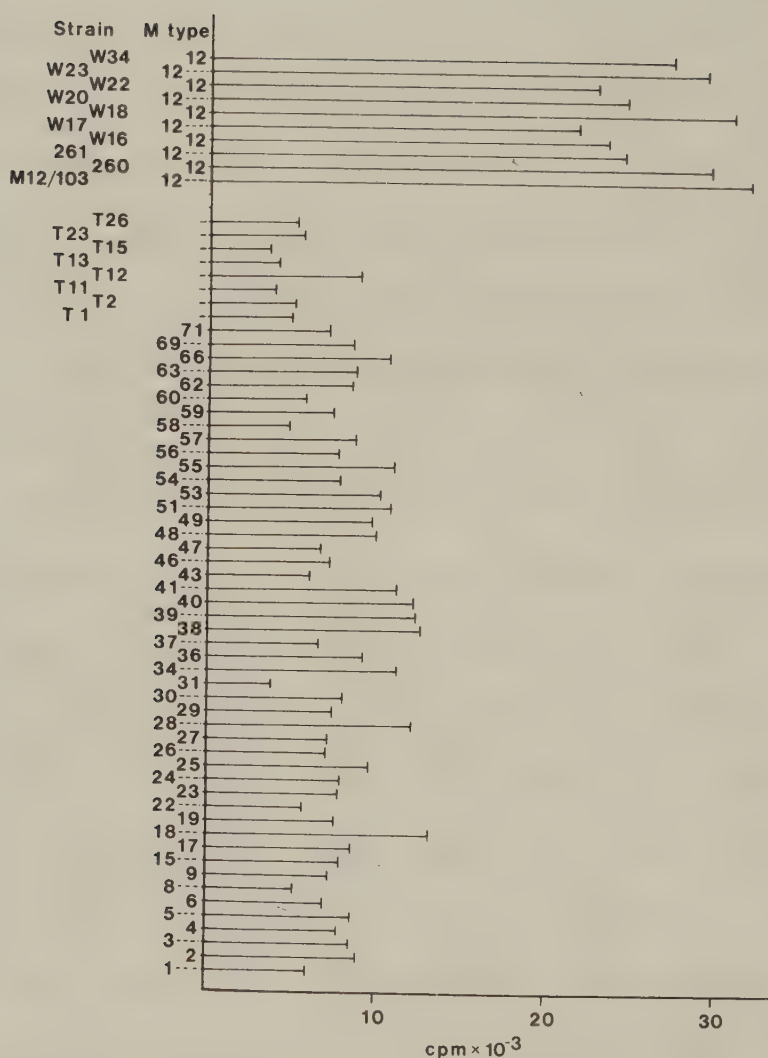


Figure 2: Binding of absorbed anti M12 antiserum to 10 homologous M types, 48 heterologous M types and 8 M negative strains. The data are presented as counts per minute.

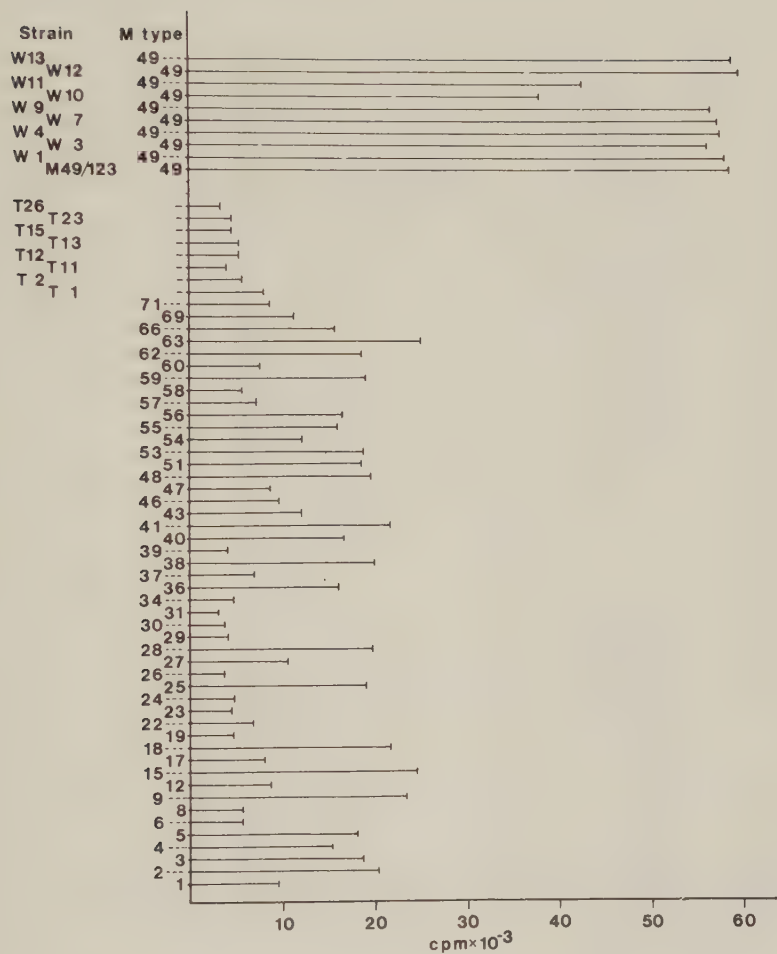


Figure 3: Binding of absorbed anti M49 antiserum to 10 homologous M types, 48 heterologous M types and 8 M negative strains. The data are presented as counts per minute.

Table 1: Binding of anti-M12 antiserum to one homologous and one heterologous group A streptococcal strain. The anti- M12 antiserum was submitted to affinity chromatography on Sepharose 4B coupled with M12 protein prior to binding experiments. Glycine-Sepharose was used as control.

M type	Antibodies absorbed with		Antibodies eluted from	
	M12-Sepharose	Glycine-Sepharose	M12-Sepharose	Glycine-Sepharose
12	10.1 ⁺ 0.2	22.9 ⁺ 1.4	1.1 ⁺ 0.1	0
15	3.4 ⁺ 0.9	3.4 ⁺ 0.2	0	0.3 ⁺ 0.2

1) For details see text

2) The data are presented as corrected counts per minute $\times 10^{-3}$ $\pm$ SD (counts per minute with anti M12 antiserum minus counts per minute with control consisting of normal rabbit serum)

Titration of Antisera, Bacteria and ^{125}I Protein A

Increasing dilutions of absorbed anti-M 49 antiserum was added to a M type 49 group A streptococcus (1×10^8 bacteria). Radiolabelled protein A (approximately 1×10^5 cpm) was added in a second step. The results are presented in Figure 4A and shows a dose-dependent binding of antibodies to the bacteria. In a second series of experiments varying amounts of radiolabelled protein A was added to a M type 49 group A streptococcus (1×10^8 bacteria) preincubated with absorbed anti-M 49 antiserum diluted 1/100 in PBS-BSA. Figure 4B shows the dose-dependent binding of protein A to the streptococci coated with antibodies.

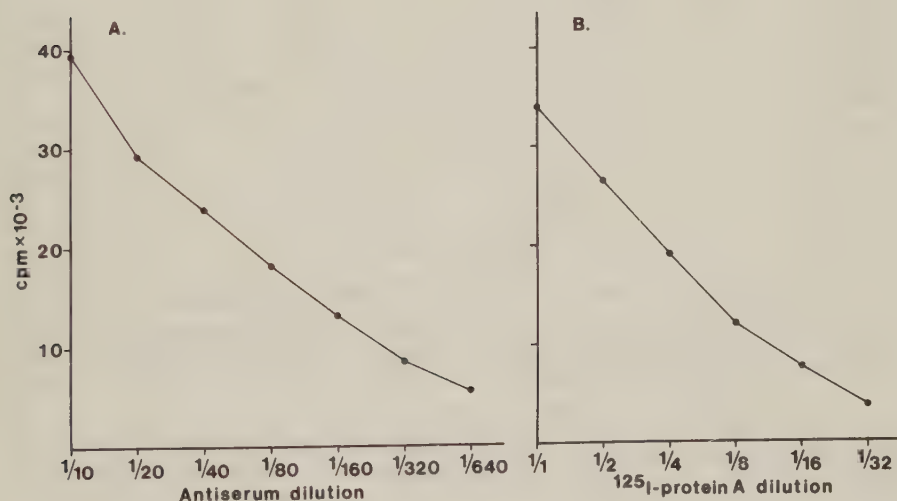


Figure 4: (A) Binding of absorbed anti M49 antiserum to a M type 49 strain at varying antiserum dilutions (B) Binding of varying amounts of ^{125}I labelled protein A to a M type 49 strain pretreated with absorbed anti M49 antiserum. The undiluted ^{125}I -protein A solution contained $25 \mu\text{g}$ protein A per ml.

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One hundred μ l absorbed anti-M49 antiserum diluted 1/100 in PBS-BSA was added to varying numbers of M type 49 streptococci. The total number of bacteria was kept constant by addition of a Escherichia coli. Figure 5 shows the binding capacity of the two M type 49 strains as a function of the number of streptococci.

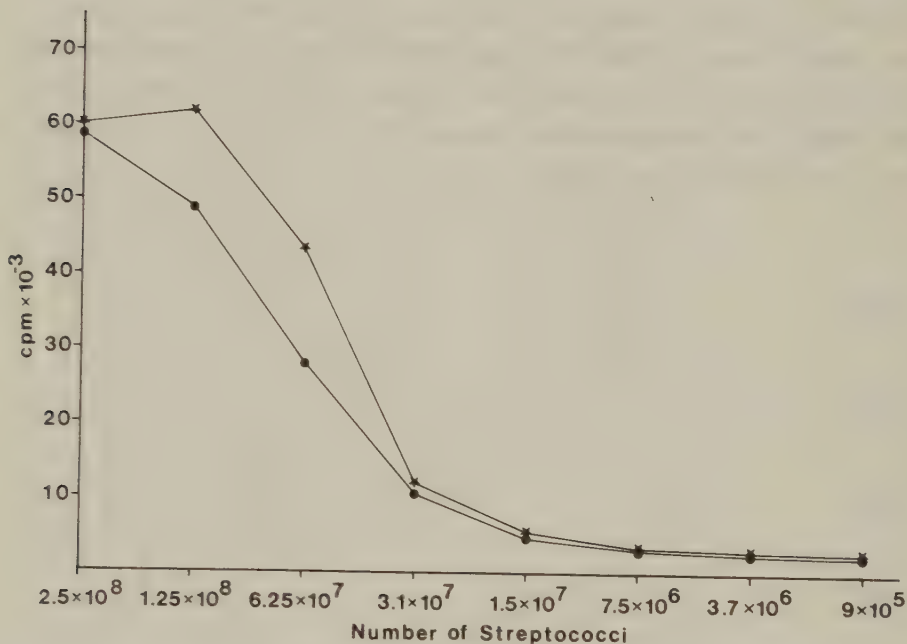


Figure 5: Binding of anti M49 antiserum to two M type 49 strains as a function of number of streptococci. The total number of bacteria was kept constant by addition of a Escherichia coli. Symbols: * Strain W1, Strain W11.

Intra-Assay Variation

Two group A streptococcal strains (M type 49 and 54) were tested 10 times on the same occasion for binding of absorbed anti-M antiserum to their surfaces. The imprecision, expressed as coefficient of variation in per cent was 2.3 and 3.9 respectively.

DISCUSSION

A simple and reliable method for the recognition of M types of group A streptococci would facilitate epidemiological and clinical studies on streptococcal diseases. The typing of M antigen is usually done by the Lancefield capillary precipitin test (2) or by the Ouchterlony immunodiffusion assay (3, 8) using acid extracted M protein and specific rabbit antisera. Several other methods have been described for typing group A streptococci (1). Quantitative or semiquantitative assays for M antigen in Lancefield extracts have been based on the capillary precipitin technique (9, 10), immunodiffusion in agar (11) and crossed immuno-electrophoresis (12). In the present study a new method for typing of group A streptococci is described. The assay is performed on whole bacteria with absorbed rabbit anti-M antisera produced against whole heat-killed cells. Staphylococcal protein A is used in a second step as an anti-antibody reagent (13). By this method one can circumvent the tedious and time consuming M protein extraction procedure. We have tested two absorbed anti-M antisera which both gave an unequivocal reaction with the homologous serotypes and only minor cross-reactions with 48 different heterologous serotypes and 8 M negative strains (Figures 2 and 3). The type-specificity of the M12 reaction was further confirmed by the removal of type-specific antibodies after absorption of the M12 antiserum with purified M protein coupled to Sepharose 4B (Table 1).

One of the problems frequently encountered with antisera prepared against whole heat-killed cells is the difficulty in removing cross-reacting antibodies. The absorption of antisera often leads to a considerable loss of type-specific antibodies. We have, how-

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ever, not encountered this problem when absorbing the antisera for use in the described radioimmunoassay. Both antisera could be diluted at least 100 times and still retain enough type-specific antibodies to be able to discriminate homologous from heterologous M types (Figure 1) One absorption with a heterologous streptococcal strain was sufficient to eliminate major cross-reacting antibodies from the antisera. Kronvall et al (14) have studied the the nonimmune binding of human serum IgG to bacteria. They have estimated that one absorption with an equal volume of group A streptococci and undiluted serum would reduce the IgG concentration of human serum by about fifteen per cent. The effect of absorption on rabbit serum IgG would be even less since rabbit IgG shows a lower degree of nonimmune binding to most group A streptococci than human IgG (15). These data are compatible with the results obtained in our assay. A discrepancy in the titer of M type-specific antibodies measured by precipitin test and by immunofluorescence has previously been demonstrated (16) and it was suggested that the precipitin test results are not truly indicative of the titer of type-specific antibodies in an antiserum. This could explain the surprising finding that a simple absorption and dilution is sufficient to obtain a type-specific reagent for use in our assay whereas the same absorption procedure might not be feasible for absorption of antisera used in other M antigen assays.

In the present investigation the bacteria were grown in Todd-Hewitt broth overnight and a suspension containing 1×10^9 bacteria per ml in PBS was made. From this suspension 100 μ l was used in the test. Preliminary results indicate a possibility to perform the test on bacteria taken directly from a blood agar plate and suspended in PBS. This would further simplify the typing procedure.

Different methods for quantitation of M antigen have been described (9-12). These methods all involve the extraction of M antigen from the bacteria. With the method described in this paper a semi-quantitative estimation of M antigen in situ is possible (Figure 5). This method has been utilized to investigate the surface properties of group A streptococci (17) and could be of interest in the study of host-parasite interactions.

The results presented in this paper indicate that the described radioimmunological method is a simple and accurate technique for M type identification of group A streptococci and offers valuable tool for the study of M antigen in situ.

ACKNOWLEDGEMENTS

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REFERENCES

1. Fox, E.N. : M proteins of group A streptococci. *Bacteriological Reviews* 1974, 38:57-86.
2. Swift, H.F., Wilson, A.T., Lancefield, R.C. : Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. *Journal of Experimental Medicine* 1943, 78:127-133.
3. Michael, J.G., Massel, B.F. : Use of unabsorbed antisera in gel diffusion for grouping and typing of hemolytic streptococci. *Journal of Laboratory Clinical Medicine* 1965, 65: 322-328.

4. Fox, E.N. : Measurement of streptococcal antigen synthesis with fluorescent antibody. *Proceedings of the Society for Experimental Biology* 1962, 109:577-579.
5. Kühnemund, O., Havlicek, J., Knöll, H., Sjöquist, J. : M antigens of group A streptococci isolated by means of immunochromatography. *Biochemical and serological properties. Immunobiology* 1981, 159: 244-255.
6. Cuatrecasas, P. : Protein purification by affinity chromatography *Journal of Biological Chemistry* 1970, 245:3059-3065.
7. Hunter, W.H., Greenwood, F.C. : Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature* 1962, 194:495-496.
8. Rotta, J., Krause, R.M., Lancefield, R.C., Everly, W., Lackland, H. : New approaches for the laboratory recognition of M types of group A streptococci. *Journal of Experimental Medicine* 1971, 134:1298-1315.
9. Brock, T.D. : Effect of antibiotics and inhibitors on M protein synthesis. *Journal of Bacteriology* 1963, 85:527-531.
10. Cohen, J.O., Pine, L. : Quantitative aspects of the M protein capillary precipitin test. *Applied Microbiology* 1968, 16:122-127.
11. Becker, C.G. : Enhancing effect of type specific antistreptococcal antibodies on the emergence of streptococci rich in M protein. *Proceedings of the Society for Experimental Biology* 1967, 124:331-335.
12. Eliasson, I., Svensson, M.-L., Ramstorp, G., Schalén, C., Christensen, P. : Quantitation of M antigen in Lancefield extracts of group A streptococci type 12 using electro-immunoassay. *Acta Pathologica Microbiologica et Immunologica Scandinavica Section B* 1980, 88:299-302.
13. Goding, J.W. : Use of staphylococcal protein A as an immunological reagent. *Journal of Immunological Methods* 1978, 20: 241-253.
14. Kronvall, G., Simmons, A., Myhre, E.B., Jonsson, S. : Specific absorption of human serum albumin, immunoglobulin A, and immunoglobulin G with selected strains of group A and G streptococci. *Infection and Immunity* 1979, 25:1-10.

15. Myhre, E.B., Kronvall, G. : Heterogeneity of gram-positive cocci: description of three major types of receptors for human immunoglobulin G. *Infection and Immunity* 1977, 17:475-482.
16. Karakawa, W.W., Borman, E.K., McFarland, C.R. : Typing of group A streptococci by immunofluorescence. I. Preparation and properties of type 1 fluorescein-labeled antibody. *Journal of Bacteriology* 1964, 87:1377-1382.
17. Miörner, H., Havlicek, J., Kronvall, G. : Surface characteristics of group A streptococci with and without M-protein. *Acta Pathologica Microbiologica et Immunologica Scandinavica* (B), in press.

ON THE INTERACTION BETWEEN β_2 - MICROGLOBULIN AND GROUP A STREPTO- COCCI WITH SPECIAL REFERENCE TO STREPTOCOCCAL M PROTEIN

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SUMMARY

β_2 -microglobulin (β_{2m}) was found to interact with many group A streptococcal strains. The interaction appears to require multi-point attachment since monomeric β_{2m} in solution showed no binding, whereas both β_{2m} monomers bound to liposomes, and β_{2m} in aggregates, showed affinity for the bacteria. Aggregated HLA antigens (-A, -B, and -C), and aggregated β_{2m} exhibited the same binding patterns when tested in binding experiments with various group A streptococcal strains. Furthermore β_{2m} aggregates in excess completely blocked the binding of aggregated HLA antigens, thereby demonstrating that β_{2m} is able to interact with streptococcal surface structures also when it is part of the HLA antigen complex. M protein-positive group A streptococcal strains were found to bind significantly more β_{2m} than M protein negative variants of these strains. It was also found that the streptococcal surface structures responsible for β_{2m} were extremely heat-stable a well-known characteristic of M proteins. Moreover, purified M 12 protein inhibited the binding of radiolabelled β_{2m} aggregates to whole streptococci, and in gel filtration and affinity chromatography experiments, the M 12 protein was found to interact with β_{2m} . These various data suggest that the interaction between β_{2m} and group A streptococci is mediated by M protein. Since it has been reported that lipoteichoic acid (LTA), a constituent of the streptococcal cell wall, forms complexes with M protein at the bacterial cell surface, we analyzed the possible significance of LTA for the binding of β_{2m} . However, LTA did not influence the interaction between β_{2m} and streptococci, suggesting that the binding of β_{2m} to streptococcal M protein represents a pure protein-protein interaction. In vivo such an interaction could be established between infecting streptococci and host cells. Among 45 strains of different M types large differences in

β_2 m-binding were recorded, whereas among 60 strains of the classical nephritogenic M types 12 and 49, all were highly β_2 m-reactive, which points towards a role for β_2 m in streptococcal pathogenicity.

INTRODUCTION

β_2 -microglobulin (β_2 m) is a small protein (Mw, 11.800) devoid of carbohydrate that was originally isolated from the urine of patients with tubular kidney damage (1). The molecule is synthesized by all cells and is also expressed at the surface of all cells as the light chain of major histocompatibility complex (MHC) class I antigens (HLA-A, -B, and -C antigens in man ; H2K, -D, -L, and -R antigens in mouse, etc.). Furthermore, β_2 m has been reported to participate in various cell-cell interactions. Structurally β_2 m is closely related to immunoglobulins (Ig) which stimulated us to investigate whether β_2 m and Ig also have other properties in common (for β_2 m references see review, ref. 2). Ig show affinity for protein A of staphylococci and cell surface structures of many streptococcal strains (3, 4). Aggregated, but not monomeric, β_2 m was indeed found to bind to many group A, B, C, and G streptococcal strains (5, 6). Surprisingly the binding of β_2 m was not correlated to IgG-binding despite the structural similarities. Moreover, β_2 m-binding was not inhibited by IgG and β_2 m-uptake was markedly trypsin-sensitive whereas IgG-binding was much more trypsin-resistant (5). Different streptococcal structures thus appeared accountable for β_2 m- and IgG-binding. Since β_2 m is present on cell surfaces the interaction with streptococci could be of importance to streptococcal pathogenicity. This led us to study the β_2 m-binding structures in more detail. Our interest was early focused on the antiphagocytic M protein of group A streptococci (7). Situated at the bacterial surface these fibrous pro-

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teins render group A streptococci a "hairy" appearance on electron micrographs (8). Like the β_{2m} -binding structures M proteins are trypsin sensitive (9). M protein-positive strains of group A streptococci bound much more β_{2m} than did M protein-negative variants of these strains (10), and analysis in the electron microscope showed binding of ferritin-labelled β_{2m} aggregates to the filamentous structures typical of M protein (11). These data suggested that β_{2m} interacts with the streptococcal surface via M protein.

β_{2m} is a molecule with multiple relations to the immune system and M proteins represents well-characterized (12, 13) streptococcal cell wall proteins. The aim of the present investigation was to further analyze the interaction between β_{2m} and group A streptococci with special reference to M protein.

MATERIALS AND METHODS

Bacterial strains

Group A streptococcal strains of 45 different M types were obtained by the courtesy of Dr. J. Havlicek at the WHO Collaborating Centre for Reference and Research on Streptococci, Institute of Hygiene and Epidemiology, Prague, Czechoslovakia. These strains are able to grow in fresh human blood for at least seven generations. Dr. Havlicek also provided seven so-called M protein-negative strains (T1, T2, T11, T12, T13, T15, T26), which do not grow in fresh human blood from at least two donors. Nine different M type 12 strains and 14 M type 49 strains were kindly provided by the late Dr. L. W. Wannamaker, University of Minnesota School of Medicine, Minneapolis, USA, and 36 M 12 strains were obtained from clinical specimens sent to the Clinical Microbiology Laboratory, University Hospital, Lund, Sweden. Furthermore three group A

strains with M type 1, 12, and 14 and M protein-negative variants of these strains were used: M type 1, T1V without (1-) and with M protein (1+) were originally from Dr.E.H. Beachey; M type 12, NY5 strain without (12-) and with (NY5 strain after mouse passages; code E14/51RB/21/2) M protein (12+) were Dr. R. Lancefield's reference strain; M type 14 strains (14-:33RS84, 14+: PHLS Lowe) were from Dr.W.Hryniewicz, Warsaw. As a negative control, finally, we used the Staphylococcus epidermidis strain L603.

Strains were stored at -70°C in Todd-Hewitt broth supplemented with 20% fetal calf serum, and on blood agar plates at 4°C . Bacteria were inoculated in Todd-Hewitt broth and incubated for 15-18 h at 37°C followed by two washings in phosphate buffered saline (PBS; 0.03 M sodium phosphate 0.12M NaCl, pH 7.2). The optical density at 620 nm was determined and the bacterial concentration was calculated from a standard curve and adjusted to 2×10^9 organisms per ml.

Proteins

Human $\beta_2\text{m}$ was purified from the urine of patients with tubular proteinuria. As compared to the original procedure (1) a simplified purification schedule was used (2). Highly purified papain-solubilized HLA-A, -B, and -C antigens (subsequently referred to as HLA antigens) were kindly provided by Dr. Per A. Peterson, Uppsala. The isolation procedure has been described (14). M type 12 protein was extracted by phage-associated lysin and purified as previously reported using immunochromatography on immobilized type-specific opsonizing antibodies as the final purification step (15). M12 protein used in analytical gel filtration experiments (Fig. 2) was first radiolabelled and separated on Sephadex G-200. Material from the top fraction of the resulting major peak was rechromatographed, alone and together with aggregated $\beta_2\text{m}$, on Sephadex G-200 as shown in Fig.2. α_1 -microglobulin, finally, was

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isolated as previously described (16).

Preparation of β_2m liposomes

Large, unilamellar liposomes (approx. 0.2 μ m diameter) were prepared from a mixture (molar ratio 5:1:2.5:1.5) of phosphatidylcholine, phosphatidylinositol, cholesterol and a semi-synthetic glycolipid (phosphatidyl-ethanol-lactobionamide, see ref 17) using a reversed-phase evaporation technique (18) followed by passage through a polycarbonate filter (0.2 μ m pore diam). Monomeric unlabelled or ^{125}I -labelled monomeric β_2m was covalently linked to aldehyde groups generated on the outer surface of the liposomes, essentially as described (19). In brief, liposomes (10 mol lipid) were treated with 50 mM sodium periodate (pH 5.5) for 2 h, passed through a Sephadex G-50 column in 0.12 M NaCl - 20 mM borate (pH 8.4) and immediately mixed with unlabelled or ^{125}I -labelled β_2m (1 mg). Protein-liposome links were stabilized by reduction with 25 mM sodium cyanoborohydride. The β_2m liposomes thus obtained were freed from non-coupled protein by flotation through 10% (w/v) of dextran T-40 at 100 000xg for 20 min. The recovery of liposomes was better than 90 %, but the yield of coupled protein (determined with ^{125}I -labelled β_2m) was consistently below 5%. Attempts to increase the coupling efficiency have not been successful. However, coupled β_2m was firmly attached to the liposomes since it remained quantitatively associated during renewed flotation through dextran. The density of β_2m molecules attached to the liposomes was estimated to $1.3 \times 10^3 / \mu m^2$. This is of the same magnitude as compared to human lymphocytes which carry $10^3 - 10^4$ β_2m molecules/ μm^2 (20).

Radiolabelling and aggregation of proteins

250 μ g β_2m and 100 μ g HLA antigens were labelled with 0.4 and 0.2 mCi ^{125}I respectively, according to the chloramine-T method (21). The radiolabelled proteins were dialyzed against 0.15 M phosphate

buffer, pH 7.6, and aggregated with glutaric dialdehyde (22). Thus 15 μ l 0.25% glutaric dialdehyde in distilled water was added to 200 μ g β_2m in 50 μ l phosphate buffer, and to 45 μ g radiolabelled HLA antigens in 100 μ l phosphate buffer was added 25 μ l 0.25% glutaric dialdehyde in distilled water. The reaction mixtures were incubated at room temperature over night and then subjected to gel filtration on Sephadex G-200 in PBS containing 0.02% sodium azide, 0.05% Tween 20 and 0.02 M glycine. Aggregates corresponding to the peak fractions of the V_0 material were used in binding experiments. The aggregation of HLA antigens could theoretically dissociate the molecule into its two chains. If true this could mean that the postulated HLA aggregates in fact consisted of aggregates of dissociated β_2m and/or HLA heavy chains. Therefore we analyzed the aggregates with a rabbit antiserum raised against purified HLA antigens (kindly provided by Dr. Per A. Peterson, Uppsala). The antiserum was first absorbed with Sepharose 4B-coupled human β_2m . This absorbed serum, specific for the heavy HLA chain, and a rabbit antiserum against human β_2m , both immunoprecipitated the assumed HLA aggregates thus demonstrating that the aggregates contained both β_2m and HLA heavy chains. This strongly suggests that our aggregation method actually did produce aggregates of intact HLA antigens.

Unlabelled aggregates of β_2m were prepared by adding 100 μ l 0.25% glutaric dialdehyde in distilled water to 2.5 mg β_2m in 500 μ l 0.15 M phosphate buffer, pH 7.6. After incubation at room temperature over night the resulting aggregates were separated and pooled as described above.

Binding assay

Overnight broth cultures of bacterial strains were washed twice in PBS containing 0.02% $NaNO_3$ (PBSA) and the bacterial concentration adjusted to 2×10^9 organisms/ml PBSA containing 0.05% Tween 20

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(PBSAT). Bacterial suspensions (200 μ l) were incubated with radio-labelled (monomeric or aggregated) β_2 m or HLA antigens (0.1 μ g corresponding to about 10^4 cpm) at room temperature for 60 minutes. Two ml of PBSAT were then added and the bacteria were sedimented by centrifugation at 2000 $\times$ G for 10 minutes. The radioactivity of the pellet was measured in a gamma counter and the binding expressed as percentage radioactivity remaining in the pellet. In binding experiments with liposomes we incubated 4×10^8 bacteria in PBS with 25 μ l radiolabelled β_2 m liposomes (5×10^3 cpm) at room temperature for 60 minutes. Two ml PBS was added and the subsequent procedure was the same as for β_2 m aggregates.

To obtain maximal sensitivity in inhibition experiments various amounts of bacteria were tested for binding of a constant amount of aggregated β_2 m or β_2 m liposomes. Depending on the strain investigated, bacterial dilutions containing between 4×10^6 and 2×10^8 bacteria/100 μ l were used. Aggregated unlabelled β_2 m (10-100 μ g), M12 protein (20 μ g), unlabelled β_2 m liposomes (10^{10} liposomes), all diluted in 25 μ l PBS, or only 25 μ l PBS, was added to the tubes which were incubated at 37°C for 30 minutes. Radiolabelled aggregated β_2 m or radiolabelled β_2 m liposomes were then added and the binding assay was performed as described above.

Temperature dependence of β_2 m-binding

Three M type 12 group A streptococcal strains, in 1% suspensions in PBS, were heated for 10 minutes at 60°C, 80°C, 85°C, 90°C, 95°C, and 100°C. The cells were then washed and used in binding experiments with radiolabelled β_2 m aggregates.

Affinity chromatography

M12 protein and α_1 -microglobulin were CNBr-coupled (23) to Sepharose 4B (1 mg protein/ml gel). Pasteur pipettes were plugged with glas wool and packed with 0.5 ml gel. Samples of radiolabelled

aggregated β_2m (4×10^5 cpm in 200 μ l PBSAT) were allowed to drain into the gels. The columns were incubated at room temperature for 3 h and then washed with 5x1 ml PBSAT or until no more radioactivity was eluted. Absorbed material was finally eluted with 5x1 ml 0.1 M glycine buffer, pH 2.0.

Determination of cell surface lipoteichoic acid (LTA)

A rabbit antiserum raised against Lactobacillus casei -LTA (kindly provided by Dr. K.W.Knox, Sydney, Australia) was used to determine surface associated LTA. Normal rabbit serum was used as a control. The test was performed as previously described (24). Fifty μ l bacterial suspension containing 5×10^7 organisms was added to 100 μ l anti-LTA diluted 1/100 in PBS containing 0.1% bovine serum albumin (PBS-BSA). After incubation at 37 C for 40 minutes, the bacteria were washed once in PBS-BSA and deposited by centrifugation. Staphylococcal protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) labelled with ^{125}I by the chloramine-T method was added to each tube. The mixtures were incubated for another 40 minutes at 37°C, washed once in PBS-BSA and centrifuged. Supernatants were discarded and the radioactivity of the bacterial pellets was measured in a gamma counter.

RESULTS

Binding of β_2 -microglobulin to group A streptococcal strains

A total of 45 group A reference streptococcal strains of different M serotypes were used in binding experiments with monomeric and glutaraldehyde aggregated β_2m . None of the strains bound more than 2% of monomeric radiolabelled β_2m . Results obtained with aggregated β_2m are shown in Fig.1. In this case 7 strains bound more than 50%, 23 strains 10-50%, and 15 strains bound less than 10% of the added radiolabelled β_2m .

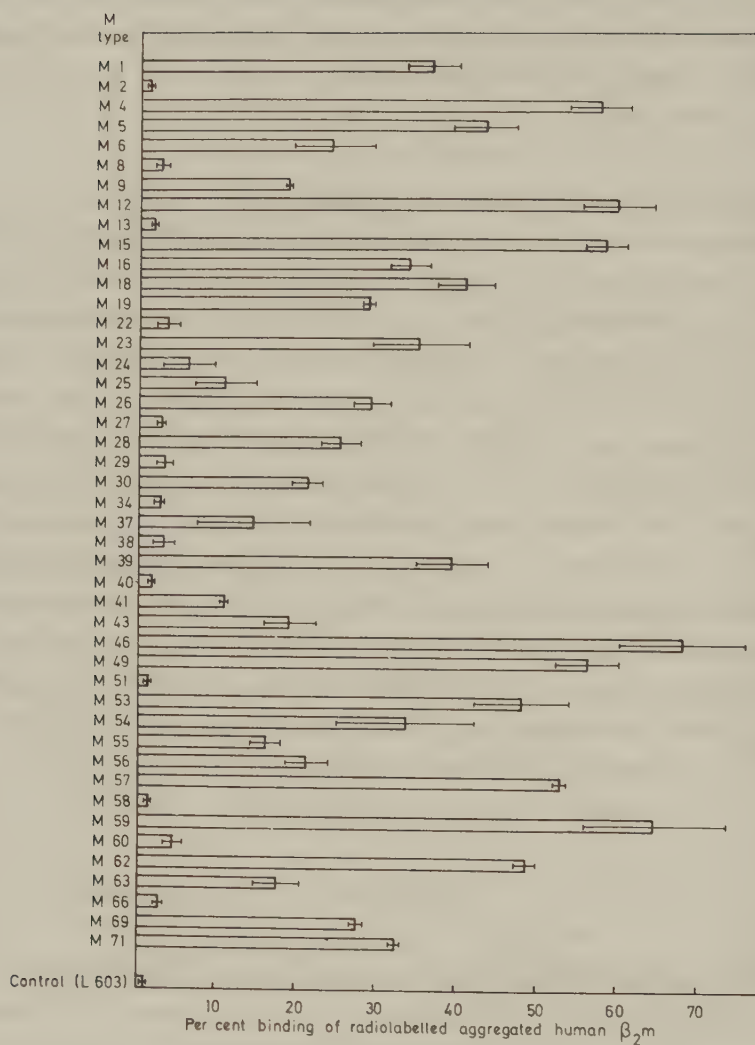


Figure 1. Binding of radiolabelled and aggregated β_2m to group A streptococcal strains of different M types. Mean values $\pm$ SEM are indicated by columns and bars.

The finding that monomeric β_{2m} does not show any affinity for streptococci is disturbing. However, in vivo β_{2m} is situated at the surface of all nucleated cells which could mean that in solution β_{2m} aggregates are more physiological than β_{2m} monomers. Thus it seemed possible that liposomes carrying monomeric β_{2m} might reflect the in vivo situation even better. However, it is not possible to incorporate β_{2m} into liposomes through hydrophobic interactions, since β_{2m} is not an integral membrane protein. To circumvent this problem unlabelled or radiolabelled monomeric β_{2m} was covalently bound to a glycolipid part of the lipid bilayer. These liposomes were then tested in binding experiments with three M protein-carrying group A strains and M protein-negative variants of these strains. For comparison, the binding of aggregated β_{2m} was tested in parallel. Both aggregates and liposomes were found to have affinity for the M protein-positive strains, and although the M protein-negative strains also showed some binding of β_{2m} , the results demonstrate that the presence of M protein is of major importance for the interaction (Table I). Binding of radiolabelled β_{2m} liposomes to the strains (including the low reactivity seen with M protein-negative strains) was completely inhibited both by non-labelled β_{2m} -liposomes and by non-labelled β_{2m} aggregates added in excess. Monomeric β_{2m} could not block the binding, which suggest that a multipoint attachment is necessary and that the affinity for β_{2m} monomers is low. However, the results demonstrate that β_{2m} liposomes and β_{2m} aggregates interact with the streptococcal cell wall in a comparable way.

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Table I. Binding of β_2m to three group A streptococcal strains of M type 1, 12, 14 and M₂ protein-negative variants of these strains. The uptake of liposomes carrying radiolabelled monomeric β_2m , and the uptake of glutaraldehyde aggregates of radiolabelled β_2m were analyzed in parallel experiments.

Strain M type	No	Percentage binding (mean $\pm$ SEM)	
		β_2m liposomes	β_2m aggregates
1+	T1V	17.3 $\pm$ 3.8	68.3 $\pm$ 3.1
1-	T1V	8.1 $\pm$ 1.8	4.5 $\pm$ 1.3
12+	NY5	26.3 $\pm$ 4.1	83.8 $\pm$ 5.7
12-	NY5	10.2 $\pm$ 2.2	20.2 $\pm$ 7.0
14+	33RS84	28.0 $\pm$ 5.2	49.6 $\pm$ 4.3
14-	PHLS Lowe	7.7 $\pm$ 2.5	4.8 $\pm$ 1.9
Control strain	L603	3.5 $\pm$ 1.2	1.4 $\pm$ 0.3

β_2 -microglobulin, HLA antigens, and group A streptococci

At the cell surface β_2m is non-covalently associated with HLA antigens. If cell-bound β_2m molecules interact with streptococcal surface structures in vivo, it seems reasonable that HLA-associated β_2m would show affinity for these structures. We therefore analyzed the binding of monomeric and aggregated HLA antigens to 25 different group A streptococcal strains (Prague collection). Analogous to the finding with monomeric β_2m , monomeric HLA antigens showed no affinity for the examined strains whereas aggregated HLA antigens did. The binding patterns for HLA and β_2m aggregates were also found to be very similar (Table II). Moreover, two of the strains (M type 4 and 12) were used in inhibition experiments and in both cases unlabelled β_2m aggregates in excess completely blocked the binding of radiolabelled aggregated HLA antigens. The results demonstrate that β_2m has affinity for the streptococcal cell wall also when associated with the heavy HLA chain and the need for aggregates once again suggests that a multipoint attachment is required.

Table II. Binding of radiolabelled aggregates of β_2m and HLA antigens to 20 group A streptococcal strains of different M type and 5 M protein-negative strains (T1 etc).

Strain (M type)	Binding of radiolabelled aggregates (%)	
	β_2m	HLA
M1	36.8 $\pm$ 3.3*	20.1 $\pm$ 2.4
M4	57.9 $\pm$ 3.9	64.4 $\pm$ 3.0
M8	2.7 $\pm$ 0.9	3.4 $\pm$ 0.5
M12	60.3 $\pm$ 4.6	25.5 $\pm$ 6.1
M13	1.8 $\pm$ 0.1	4.1 $\pm$ 0.2
M15	58.8 $\pm$ 2.7	27.6 $\pm$ 4.3
M23	35.3 $\pm$ 6.1	56.3 $\pm$ 7.2
M24	6.3 $\pm$ 3.3	3.0 $\pm$ 0.4
M25	10.9 $\pm$ 4.0	8.4 $\pm$ 0.2
M34	2.8 $\pm$ 0.6	7.9 $\pm$ 0.1
M37	14.5 $\pm$ 7.2	3.8 $\pm$ 1.2
M46	68.5 $\pm$ 7.8	40.1 $\pm$ 1.8
M53	48.2 $\pm$ 6.1	53.2 $\pm$ 4.8
M54	33.7 $\pm$ 8.6	24.4 $\pm$ 3.0
M58	1.4 $\pm$ 0.1	4.7 $\pm$ 1.3
M59	64.8 $\pm$ 8.9	50.6 $\pm$ 7.3
M60	4.5 $\pm$ 1.3	4.5 $\pm$ 0.7
M62	48.7 $\pm$ 1.3	33.4 $\pm$ 8.6
M66	2.7 $\pm$ 0.6	5.8 $\pm$ 0.3
M71	32.4 $\pm$ 0.6	24.8 $\pm$ 6.0
T1	1.3 $\pm$ 0.1	1.4 $\pm$ 0.3
T2	1.6 $\pm$ 0.3	2.8 $\pm$ 0.1
T11	1.1 $\pm$ 0.1	2.2 $\pm$ 0.4
T12	1.2 $\pm$ 0.1	3.4 $\pm$ 0.3
T13	1.4 $\pm$ 0.2	1.8 $\pm$ 0.2
Control strain L603	1.0 $\pm$ 0.2	1.5 $\pm$ 0.5

*Values are mean $\pm$ SEM

β_2 -microglobulin and streptococcal M protein

Our previous investigations have indicated that M protein is the bacterial cell wall structure responsible for β_2m -binding. In order to further analyze a possible interaction between β_2m and M protein several different experimental approaches were used:

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a) Seven M protein-negative and 45 M protein-positive streptococcal strains, all from the Prague collection, were analyzed for binding of aggregated β_2m . The results were statistically analyzed with Fisher's exact probability test and the difference was found highly significant ($p = 0.00127$) which indicates that M protein is needed for β_2m -binding.

b) It is known that M proteins are extremely heat stable (25). Boiling three M type 12 group A streptococcal strains for 10 minutes did not influence their β_2m -binding capacity, demonstrating that the structures responsible for β_2m -binding also are very heat stable.

c) If the streptococcal surface structure binding β_2m is M protein, one would expect purified M protein to inhibit this binding. To test this purified M 12 protein was used in inhibition experiments with M type 4 and M type 12 streptococci. Despite a large excess of M protein only partial blocking was achieved (Table III). This can be explained by the requirement for multipoint attachment as well as by the well-known heterogeneity of M protein preparations and will be further considered in the Discussion.

Table III. Inhibition of the binding of radiolabelled aggregated β_2m and HLA antigens to M type 4 and M type 12 streptococci (Prague strains) by M type 12 protein purified from the M 12 strain. M 12 protein in PBS (or only PBS as control) was added to the bacteria prior to addition of radiolabelled β_2m or HLA antigens. For experimental details see Materials and Methods. Figures are mean $\pm$ SEM.

Strain (M type)	Per cent HLA-binding after addition of		Per cent β_2m -binding after addition of	
	PBS	M 12 protein	PBS	M 12 protein
M 4	57.4 $\pm$ 6.1	31.4 $\pm$ 2.9	68.4 $\pm$ 7.1	28.2 $\pm$ 4.8
M12	42.5 $\pm$ 3.9	19.8 $\pm$ 2.6	74.0 $\pm$ 5.1	42.1 $\pm$ 6.0

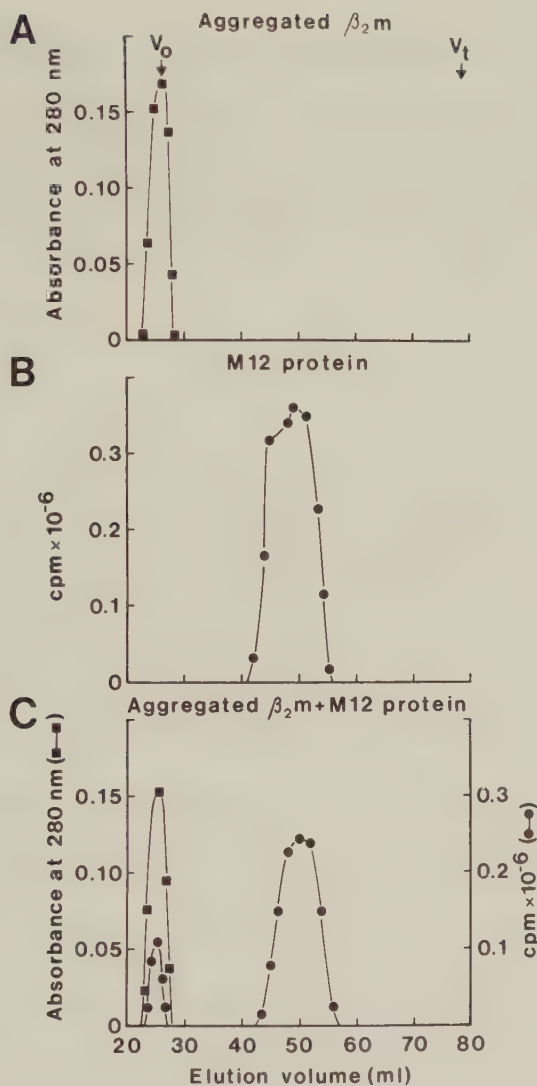


Figure 2. Interaction between β_2m and M 12 protein studied in gel filtration experiments on a G-200 superfine column (bed volume 78.5 ml). The column was eluted with 0.02 M TRIS-HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.02 % NaN_3 . 1.05 ml fractions were collected (flow rate: 1.6 ml/hr) and analyzed for absorbance at 280 nm and/or radioactivity.

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(figure 2 continued)

A: 0.3 mg aggregated unlabelled β_2m in 1.0 ml PBS was applied to the column.

B: 3.1×10^6 cpm (corresponding to 0.5 μ g protein) M 12 protein in 1.0 ml PBS was separated on the column (for a description of the radiolabelled M protein, see Materials and Methods).

C: 2.6×10^6 cpm (corresponding to 0.5 μ g protein) M 12 protein in 0.5 ml PBS was added to 0.3 mg aggregated unlabelled β_2m in 0.5 ml PBS. The mixture was incubated at 37°C for 3 hrs and then submitted to gel filtration.

d) The interaction between M 12 protein and β_2m was also studied in gel filtration experiments. Thus, radiolabelled M 12 protein was incubated with aggregated unlabelled β_2m followed by gel chromatography of the incubation mixture. Under the experimental conditions, about 20% of the radiolabelled M protein was moved to the V_0 peak which corresponds to aggregated β_2m (Fig. 2). Also in this case the incomplete interaction can be explained by M protein heterogeneity.

e) Finally, when radiolabelled aggregated β_2m was submitted to affinity chromatography on Sepharose 4B coupled with M 12 protein, 5-10% of the radioactivity was specifically bound to the column. A column of Sepharose 4B coupled with a control protein (α_1 -microglobulin) showed no β_2m -affinity (Fig. 3). Given the complexity of the experiment, where the interaction between two cell surface proteins is studied in vitro, it is not surprising that only a fraction of the β_2m is retained on the column. Thus, the result might well be influenced by the arrangement of M protein on the Sepharose beads. The experiment, together with the other data reported above, however, strongly suggest that M protein represents the surface structure of group A streptococci responsible for β_2m -binding.

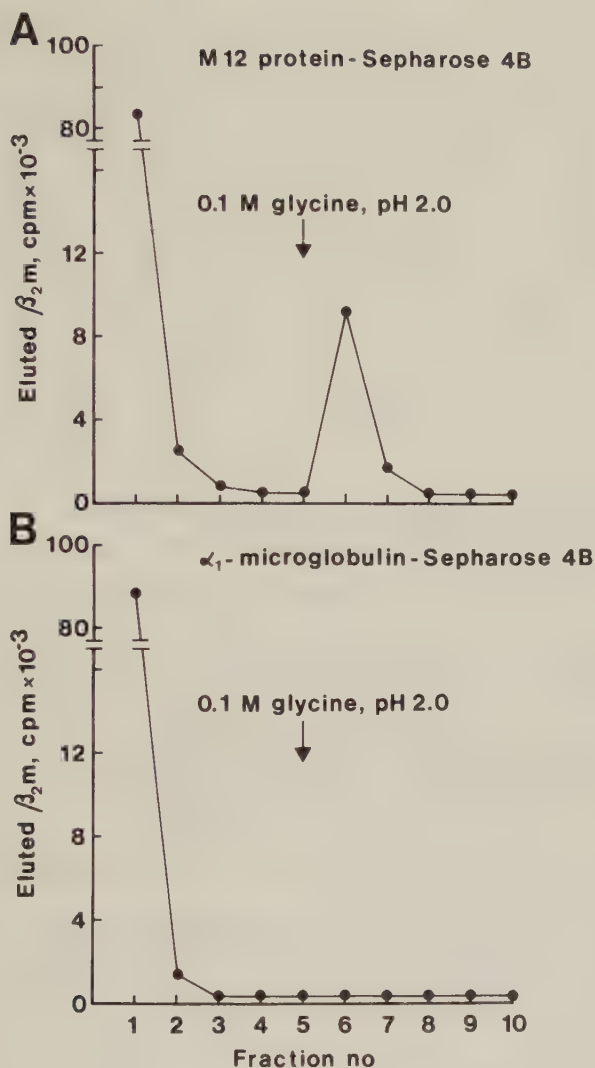


Figure 3. Affinity chromatography of radiolabelled aggregated β_2m on Sepharose 4B coupled with M 12 protein (A) or α_1 -microglobulin (B). In both cases 1.1×10^5 cpm β_2m ($1.5 \mu g$) in 0.2 ml PBSAT was submitted to the columns (0.5 ml bed volume). The columns were eluted with 5x1 ml PBSAT followed by 5x1 ml glycine buffer, pH 2.0.

The data shown in Fig. 1. indicate that streptococcal strains of different M type show a great variation in β_2 m-binding. Binding to the M 12 strain, for instance, was still >50% also when the number of bacteria used in the assay was reduced from 4×10^8 to 4×10^6 organisms whereas other M protein-carrying strains did not bind significant amounts of β_2 m even when 10^9 bacteria were used. This raises the question if β_2 m-binding differs also among group A streptococcal strains of the same M type. However, we found no evidence for this possibility. Thus, 46 M 12 and 14 M 49 strains obtained from different streptococcal epidemics in Sweden and the USA were all highly β_2 m-reactive.

Lipoteichoic acid (LTA) and β_2 -microglobulin-binding

Lipoteichoic acid (LTA) is a constituent of the cell wall of group A streptococci that has been suggested to form complexes with M protein (26). The possible significance of LTA for β_2 m-binding to streptococcal surfaces was therefore analyzed. Thus the content of cell surface LTA was determined in a number of strains and then compared to the capacity of these strains to bind β_2 m. No correlation was found between the two parameters (Fig.4). Among 28 tested strains two had a high LTA content and were also β_2 m-reactive. These strains were pretreated with sodium dodecyl sulphate (SDS) and again tested for LTA content and β_2 m-binding. In both strains β_2 m-binding was not influenced by SDS whereas LTA content decreased considerably also at low concentrations of the detergent. Figure 5 summarizes the results obtained with one of the two strains. These data and the lack of correlation between LTA content and β_2 m-binding indicate that β_2 m is bound to M protein via protein-protein interactions rather than by hydrophobic interactions between β_2 m and LTA in complex with M protein.

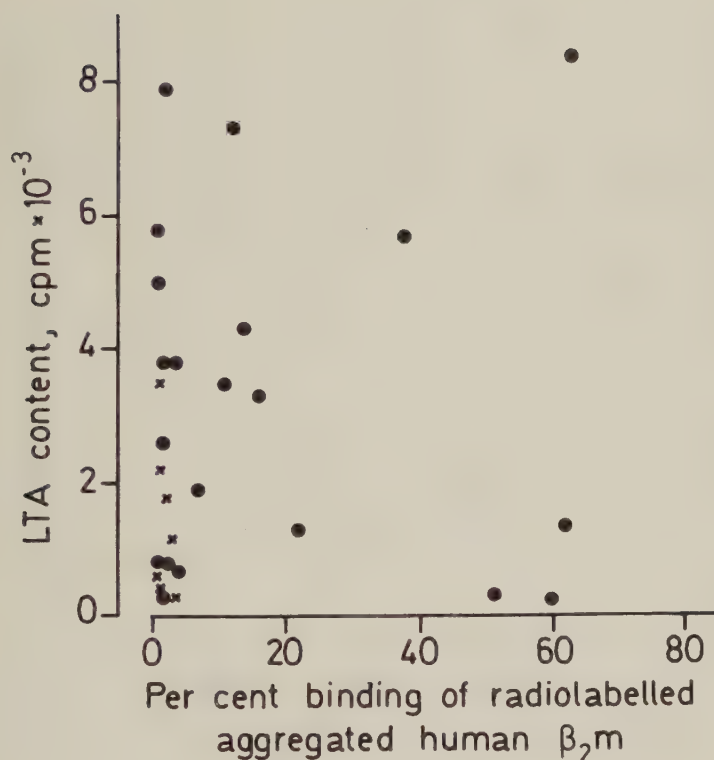


Figure 4. Lack of correlation between binding of aggregated β_2m and the content of surface LTA. Per cent uptake of β_2m of 21 M protein-positive (●) and seven M protein-negative (X) strains of group A streptococci is plotted against the content of surface LTA.

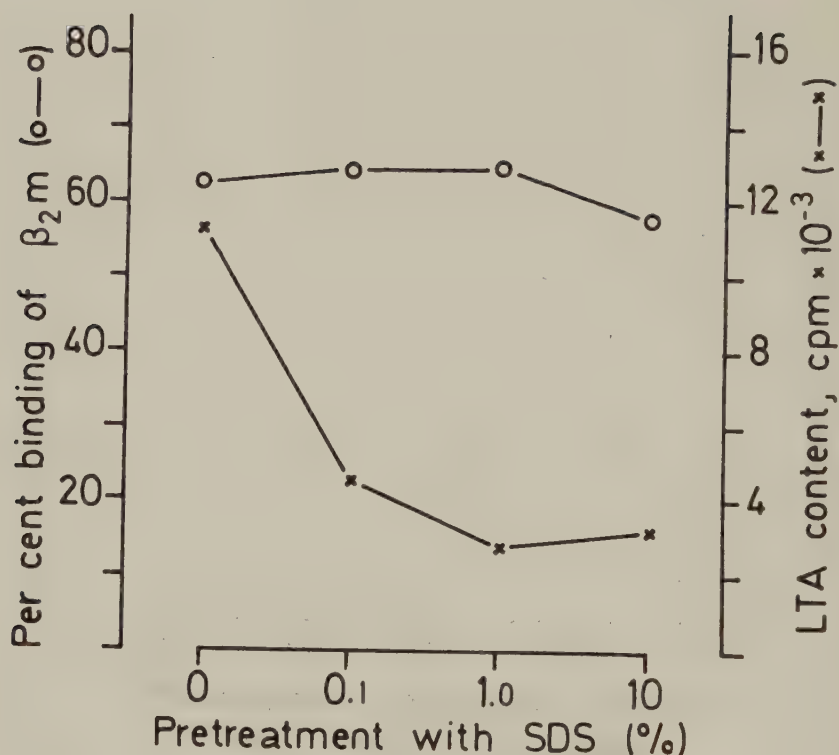


Figure 5. Group A streptococci (M type 4, Prague collection) were incubated in PBSA containing varying amounts of SDS for two h at room temperature followed by washing in PBSA. The bacteria were then used in binding experiments with aggregated radiolabelled β_2m , and their content of surface LTA was determined.

DISCUSSION

The results of the present investigation are to a large extent based on data obtained in a binding assay in which radiolabelled aggregates of β_2m show a varying degree of affinity for different group A streptococcal strains. It is therefore important to ask if our assay is likely to mimic at least some part of the surely most complex course of events taking place during infections with group A streptococci. Apart from cells of the trophoblastic layer of the placenta, all human nucleated cells seem to carry surface-bound β_2m (cf. ref. 2). In our assay aggregates of β_2m and HLA antigens are required indicating that a multipoint attachment is necessary for the binding of β_2m to streptococcal surface structures. In vivo, such an attachment could be created between cell-bound β_2m and infecting streptococci. Thus, the adherence to epithelial cells of β_2m -binding bacteria is partly inhibited if the cells are pretreated with antibody fragments directed against β_2m (27), which suggests that there is an interaction between cell-bound β_2m and streptococci. Further credence is lent to this notion by the fact, reported here, that liposomes containing monomeric β_2m show affinity for streptococci. Although β_2m liposomes may seem more physiological than β_2m aggregates we found a good correlation in the binding obtained with liposomes as compared to that obtained with β_2m aggregates. It is indeed possible that an aggregated form of β_2m is normally present on the cell surface since it has been reported that β_2m on the cell surface is polymerized by tissue-transglutaminase (28). The formation of β_2m aggregates could also be stimulated at the cell surface if streptococcal structures with affinity for β_2m are brought in close contact with host cells. With this background information we found it justified to use aggregated β_2m and HLA antigens in our binding assay. Compared to liposomes, aggregates make the assay much less

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time and β_2m consuming.

All current data support the view that β_2m is not expressed at the cell surface in free form. Apart from MHC class I antigens, β_2m has also been found non-covalently associated with other integral membrane proteins encoded by genes linked to the murine MHC, namely TL and Qa antigens (for references see ref. 2). These antigens have a restricted tissue distribution and are mainly expressed on various lymphocyte subpopulations, which is in contrast to MHC class I antigens which are found on most cells. At the surface of human cells HLA-A, -B, and -C antigens thus appear the most likely participants in an interaction with streptococci. It has previously been reported that liposome-bound HLA antigens interact with strains of Branhamella catarrhalis and Haemophilus influenzae (streptococci were not included in the study) but this interaction seemed to involve the heavy HLA chain rather than β_2m (29). In the present work we found a very close correlation between β_2m - and HLA-binding, and HLA-binding was also inhibited by β_2m . Our results favour the conclusion that β_2m in the HLA complex is able to interact with streptococcal surface structures. However, it should be remembered that the heavy HLA chain and β_2m are structurally related (30, 31), implying that also the β_2m -like domain of the heavy chain could take part in the interaction with streptococci.

Group A streptococcal strains of different M protein serotypes show considerable variation in β_2m -binding capacity (Fig.1). On the basis of the present data it is not possible to say whether the variation is due to differences in M protein structure, varying M protein density among the tested strains or other factors. Representing the cathabolic site for β_2m , high concentrations of the protein is found in the kidney (32). Among 60 strains belonging to the nephritogenic M serotypes 12 or 49, all strains were

highly β_2 m-reactive and a possible relation between β_2 m-binding and nephritogenicity is currently investigated.

As mentioned previously M protein and the streptococcal structures responsible for β_2 m-binding have properties (trypsin sensitivity and thermal stability) in common. On electron micrographs of M protein-positive and β_2 m-binding streptococci, ferritin-labelled β_2 m aggregates stained the outer layer of projections typical for M protein. Among M protein-negative strains the great majority of bacteria were lacking these fibers and showed no staining whereas a few cells carrying M protein-like fibers were also heavily stained by ferritin-conjugated β_2 m aggregates (11). These results are compatible with those obtained in this study showing that M protein-negative strains bind significantly less radiolabelled aggregated β_2 m as compared to M protein-positive streptococcal strains. In the present work we have also used purified M 12 protein to further analyze the interaction between β_2 m and streptococci. In various experimental systems we could detect a consistent but subtotal interaction between β_2 m and M protein. On the analogy of many previously described M protein preparations our M protein was also heterogeneous (15) which could be a reason for the partial binding recorded in gel filtration experiments (Fig. 2). Another explanation for the subtotal binding could be related to interaction between M protein molecules at the streptococcal cell surface (33), which may create M protein complexes with multiple β_2 m-binding sites and explain our difficulties to completely block β_2 m-binding to whole streptococci with monomeric M protein (Table III). If a high M protein density is required it could also explain the limited interaction between β_2 m-aggregates and Sepharose 4B-coupled M12 protein (Fig.3). Despite these incomplete molecular interactions we feel that the data obtained with purified M12 protein in combination with the other results described above, provide strong evidence for the suggestion that

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β_2^m interacts with streptococci via M protein.

Lipoteichoic acid (LTA) has been reported to form complexes with M protein (26). Some investigations support the view that LTA is of importance to the adherence of streptococci and that fibronectin of epithelial cell surfaces may act as the receptor for LTA (for a review see ref.34). β_2^m is water-soluble and does not interact hydrophobically with for instance liposomes (see above) and the present investigation demonstrates that β_2^m -binding to streptococci is not mediated by LTA (Fig.4 and 5). Our results are however not inconsistent with the abovementioned hypothesis that LTA is of importance for the adherence of streptococci to epithelial cells. On the contrary, such a mechanism could be needed to bring streptococcal M protein and β_2^m at the epithelial cell surface close enough to permit protein-protein interactions.

Part of the β_2^m molecule is highly evolutionary conserved (35). β_2^m -binding proteins have been detected and partly characterized in serum of various mammalian and non-mammalian species and β_2^m -binding molecules are found at the surface of mammalian and fish lymphocytes (for a review see ref.36). Moreover, *S. mansoni* schistosomula have been reported to carry receptors for human β_2^m (37). MHC class I antigens, TL antigens, and Qa antigens are all associated with β_2^m (38). These proteins are composed of domains and also β_2^m and immunoglobulins have this basic structure (39). Moreover, M proteins appear to exhibit functional domains which could be responsible for the ability of M protein molecules to adhere to each other and to epithelial cells (33). If β_2^m is bound to these regions, and if these M protein regions are structurally related to other β_2^m -binding proteins, this could mean that the interaction between β_2^m and streptococcal M protein reflects a molecular interaction of basic biological significance.

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REFERENCES

1. Berggård, I. and A.G. Bearn. 1968. Isolation and properties of a low molecular weight β_2 -globulin occurring in human biological fluids, J. Biol. Chem., 243: 4095.
2. Berggård, B., L. Björck, R. Cigén, and L. Lögdberg. 1980. β_2 -microglobulin. Scand. J. clin. Lab. Invest., 40, Suppl. 154, p. 13.
3. Forsgren, A. and J. Sjöquist. 1966. Protein A from S. aureus. I. Pseudoimmune reaction with human γ -globulin. J. Immunol. 97: 822.
4. Kronvall, G. 1973. A surface component of group A, C, and G streptococci with non-immune reactivity for immunoglobulin G. J. Immunol. 111: 1401.
5. Kronvall, G., E.B. Myhre, L. Björck, and I. Berggård. 1978. Binding of aggregated human β_2 -microglobulin to surface protein structure in group A, C and G streptococci Infect. Immun. 22: 136.
6. Schönbeck, C., L. Björck, and G. Kronvall. 1981. Receptors for fibrinogen and aggregated β_2 -microglobulin detected in strains of group B streptococci. Infect. Immun. 31: 856.
7. Lancefield, R.C. 1959. Persistence of type-specific antibodies in man following infection with group A streptococci. J. Exp. Med. 110: 271.
8. Swanson, J., K.C. Hsu, and E.C. Gotschlich. 1969. Electron microscopic studies on streptococci. I. M antigen. J. Exp. Med. 130: 1063.

9. Lancefield, R.C. 1943. Studies on the antigenic composition of group A hemolytic streptococci. I Effect of proteolytic enzymes on streptococcal cells. J. Exp. Med. 106: 525.
10. Björck, L., S.K. Tylewska, T. Wadström, and G. Kronvall. 1981. β_2 -microglobulin is bound to streptococcal M protein. Scand. J. Immunol. 13: 391.
11. Wagner, M., B. Wagner, G. Kronvall, and L. Björck. 1983. Electron microscopic localization of receptors for aggregated β_2 -microglobulin on the surface of β -hemolytic streptococci. Infect. Immun. in press.
12. Beachey, E.H., G.H. Stollerman, E.Y. Chiang, T.M. Chiang, J.M. Seyer, and A.H. Kang. 1977. Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino terminal region of type 24 M antigen. J. Exp. Med. 145: 1469.
13. Manjula, B.N., and V.A. Fischetti, 1980. Tropomyosin-like seven residue periodicity in three immunologically distinct streptococcal M proteins and its implications for the anti-phagocytic property of the molecule. J. Exp. Med. 155: 695.
14. Peterson, P.A., L. Rask, and J.B. Lindblom. 1974. Highly purified papain-solubilized HL-A antigens contain β_2 -microglobulin. Proc. Natl. Acad. Sci. USA 71: 35.
15. Kühnemund, O., J. Havlicek, H. Knöll, and J. Sjöquist. 1981. M antigens of group A streptococci isolated by means of immuno-chromatography. Biochemical and serological properties. Immunobiol. 159: 244.
16. Ekström, B. and I. Berggård. 1977. Human β_2 -microglobulin. Purification procedure, chemical and physicochemical properties. J. Biol. Chem. 252: 8048.
17. Sundler, R., and J. Wijkander. 1983. Protein-mediated inter-membrane contact specifically enhances Ca^{2+} -induced fusion of phosphatidate-containing membranes. Biochim. Biophys. Acta. 730: 391.
18. Szoka, F., and D. Papahadjopoulos. 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. USA 75: 4194.
19. Heath, T-D., B.A. Macher, and D. Papahadjopoulos. 1981. Co-

- valent attachment of immunoglobulins to liposomes via glycosphingolipids. Biochim. Biophys. Acta. 640: 66.
20. Björck, L., B. Åkerström, and I. Berggård. 1979. Occurrence of β_2 -microglobulin in mammalian lymphocytes and erythrocytes. Eur. J. Immunol. 9: 486.
 21. Greenwood, F.C., W.M. Hunter, and J.S. Glover. 1963. The preparation of ^{131}I -labelled human growth hormone of high specific radioactivity. Biochem. J. 89: 114.
 22. Avrameas, S. 1969. Coupling of enzymes to proteins with glutaraldehyd. Use of conjugates for the detection of antigens, and antibodies. Immunochemistry 6: 43.
 23. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. J. Biol. Chem. 245: 3059.
 24. Miörner, H., G. Johansson, and G. Kronvall. 1983. Lipoteichoic acid is the major cell wall component responsible for surface hydrophobicity of group A streptococci. Infect. Immun. 39: 336.
 25. Lancefield, R.C. 1928. The antigenic complex of Streptococcus haemolyticus. J. Exp. Med. 47: 91.
 26. Ofek, J., W.A. Simpson, and E.H. Beachey. 1982. Formation of molecular complexes between a structurally defined M protein and acylated or deacylated lipoteichoic acid of Streptococcus pyogenes. J. Bacteriol. 149: 426.
 27. Björck, L., S. Tylewska, T. Wadström, M. Söderström, and G. Kronvall. 1982. β_2 -microglobulin and its relation to streptococcal M protein and adherence. In: Holm, S.E., and P. Christensen (eds.) Basic concepts of streptococci and streptococcal diseases. Reedbooks Ltd., Chertsey, England, pp 220-222.
 28. Fesus, L., A. Falus, A. Erdei, and K. Laki. 1981. Human β_2 -microglobulin is a substrate of tissue transglutaminase: Polymerization in solution and on the cell surface. J. Cell. Biol. 89: 706.
 29. Klareskog, L., G. Banck, A. Forsgren, and P.A. Peterson. 1978. Binding of HLA antigen-containing liposomes to bacteria. Proc. Natl. Acad. Sci. USA. 75: 6197.
 30. Trägårdh, L., K. Wiman, L. Rask, and P.A. Peterson. 1978. Amino acid sequence homology between HLA-A, -B, -C antigens,

- β_2 -microglobulin and immunoglobulins. Scand. J. Immunol. 8: 563.
31. Orr, H.T., D. Lancet, R.J. Robb, J.A. Lopez de Castro, and J.L. Strominger. 1979. The heavy chain of human histocompatibility antigen HLA-B7 contains an immunoglobulin-like region. Nature 282: 266.
 32. Governa, M., and S. Biguzzi. 1976. β_2 -microglobulin distribution in human normal tissues. Eur. J. Immunol. 6: 830.
 33. Phillips, G.N., Jr., P.F. Flicker, C. Cohen, B.N. Manjula, and V.A. Fischetti. 1981. Streptococcal M protein: α -helical coiled-coil structure and arrangement on the cell surface. Proc. Natl. Acad. Sci. USA. 78: 4689.
 34. Beachey, E.H., and W.A. Simpson. 1982. The adherence of group A streptococci to oropharyngeal cells: the lipoteichoic acid adhesin and fibronectin receptor. Infection 10: 107.
 35. Shalev, A., A.H. Greenberg, L. Lögdberg, and L. Björck. 1981. β_2 -microglobulin-like molecules in low vertebrates and invertebrates. J. Immunol. 127: 1186.
 36. Shalev, A., L. Lögdberg, and L. Björck. 1983. β_2 -microglobulin, histocompatibility, and H-Y antigen homologues in invertebrates and early vertebrates. In: Cell Receptors and Cell Communication in Invertebrates. A.H. Greenberg, Ed. (B.A. Cinader, Series-Editor). Marcel Dekker Inc. New York, in press.
 37. Torpier, G., A. Capron, and M.A. Quaiissi. 1979. Receptor for IgG (Fc) and human β_2 -microglobulin on S. mansoni schistosomula. Nature 278: 447.
 38. Goodenow, R.C., M. McMillan, M. Nicolson, B.T. Sher, K. Eakle, N. Davidson, and L. Hood. 1982. Identification of the class I genes of the mouse major histocompatibility complex by DNA-mediated gene transfer. Nature 300: 231.
 39. Peterson, P.A., B.A. Cunningham, I. Berggård, and G. Edelman. 1972. β_2 -microglobulin - a free immunoglobulin domain. Proc. Natl. Acad. Sci. USA 69: 1697.

